ISOLATION, CHARACTERIZATION, AND EXPRESSION OF THE RAT LIVER GLUTATHIONE S-TRANSFERASE YB_1 SUBUNIT GENE

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ABSTRACT

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Thesis dated September, 1989

In situ hybridization using a cDNA probe complementary to glutathione S-transferase Yb_1 mRNA was utilized to isolate a glutathione S-transferase Yb_1 gene from a rat liver genomic library. DNA sequence analysis incorporating "gene walking" strategies revealed that the Yb_1 gene spans approximately 5.5 Kb, is comprised of eight exons separated by seven introns and is 99% homologous to the Yb_1 cDNA clone pGTA/C44. The transcription start site, which maps 29 bp downstream of the TATA element, was determined by primer extension analysis.

Gene fragments containing the Yb_1 promoter (and various deletions of the promoter region) were inserted upstream of the structural gene encoding chloramphenicol acetyl transferase (CAT). When these chimeric genes were transfected into mammalian cells, CAT assays revealed that the Yb_1 minimal promoter is contained within an 80 bp DNA fragment which contains a TATA element, a transcription initiation site and 50 bp of sequence upstream of the TATA element. Contrary to in vivo studies which have shown that Yb_1 gene activity is induced in response to phenobarbital and 3-methylcholanthrene administration, no elevation in activity was observed when mammalian hepatoma cells which had been transfected with the chimeric genes were treated with these xenobiotics. These data suggests that the regulatory element which confers xenobiotic inducibility is absent from the first 1700 bp of the Yb_1 gene.

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS ii

LIST OF TABLES iv

LIST OF FIGURES ..... v-vi

LIST OF ABBREVIATIONS vii

INTRODUCTION 1

METHODS 25

RESULTS AND DISCUSSION 49

CONCLUSION 80

REFERENCES 83

LIST OF TABLES

Table		Page
1	Glutathione S-Transferase Nomenclature	5
2	Gem Seq K/RT System Reverse Transcriptase Nucleotide Mix Formulations	36
3	Gem/Seq K/RT Klenow Nucleotide Mix Formulations	37
4	Modified Klenow Nucleotide Mix Formulations	38
5	Nucleotide Mix Formulations: Sequenase System	40
6	Sizes of Exons and Introns in the Glutathione S-transferase Yb_1 gene	59
7	Summary of Intron Sequence Homology Between the Glutathione S-transferase Yb_1 and Yb_2 Genes	60

LIST OF FIGURES

Figure		Page
1	Glutathione S-transferase activities	6
2	Reactions depicting assays for glutathione S-transferase activity.	10
3	Inducers of the glutathione S-transferases.	14
4	Metabolic pathway for the synthesis of mercapturic acids	15
5	Amidite synthesis cycle.	33
6	Polymerase chain reaction.	44
7	Partial restriction map of pGTA/C44.	50
8	Autoradiograph of in situ hybridization of plaques.	51
9	EcoRI digestion of positive λ clones on 1% agarose stained with ethidium bromide.	52
10	Southern blot analysis of λ GTA/C22.	53
11	The exon-intron structure of a glutathione S-transferase Yb_1 gene.	55
12A	Nucleotide sequence of a glutathione S-transferase Yb_1 gene.	56
12B	Nucleotide sequence of a glutathione S-transferase Yb_1 gene. (Con't)	57
13	Comparison of amino acid sequence of glutathione S-transferase Yb_1, Yb_2, Yb_3, and Yb_4 subunits.	61
14	Autoradiograph of primer extension analysis.	62
15	Partial restriction map of pGTA 2.5 K.	64
16	Autoradiograph of CAT assay using lysed human Hep G2 cells transfected with glutathione S-transferase-CAT chimeric plasmids.	65
17	Autoradiograph of CAT assay using lysed rat H5-6 cells transfected with glutathione S-transferase-CAT chimeric plasmids.	66
18	Schematic diagram of pGTA 1.9 CAT, pGTA 1.7 CAT, and pGTA 1.7 CATB.	68

LIST OF FIGURES (Con't)

Figure		Page
19	Autoradiograph of CAT assay using lysed human Hep G2 cells transfected with glutathione S-transferase-CAT chimeric plasmids.	70
20	Autoradiograph of CAT assay using lysed human Hep G2 cells transfected with glutathione S-transferase-CAT chimeric plasmids.	71
21	Schematic diagram of PCR generated Yb_1-CAT chimeric plasmids	72
22	Nucleotide sequence of 5' flanking region of the GST-Yb_1 gene.	74
23-27	Autoradiograph of CAT assay using lysed human Hep G2 cells transfected with glutathione S-transferase-CAT chimeric plasmids.	75-79

LIST OF ABBREVIATIONS

BSA	bovine serum albumin
BP	benzo(a)pyrene
CAT	chloramphenicol acetyl transferase
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EtOH	ethanol
GSH	glutathione
GST	glutathione S-transferase
Hepes	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
KAc	potassium acetate
3MC	3-methylcholanthrene
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NaOAc	sodium acetate
NH_4OAc	ammonium acetate
Pb	phenobarbital
PBS	phosphate buffered saline
Pipes	piperazine-N-N'-bis 2-ethanesulfonic acid
SDS	sodium dodecyl sulfate
SSC	sodium chloride and trisodium citrate
SSPE	sodium chloride, sodium phosphate and EDTA
Tris	tris(hydroxymethylaminomethane)

INTRODUCTION

Overview

The glutathione S-transferases play a predominant role in protection against carcinogenesis. The family of isozymes provide several detoxification mechanisms against cytotoxic drugs and carcinogens. Therefore, it is vital to obtain an understanding of the processes involved in the regulation of these enzymes.

The cDNA clones representing the Ya, Yb_1, Yb_2 and Yc mRNAs have been isolated and used to demonstrate that both the Ya and Yb subunits are induced in the livers of rats administered various xenobiotics, including 3-methylcholanthrene and phenobarbital (1). The induction has been attributed to an increase in transcriptional activity of the genes (2). The mechanism by which the glutathione S-transferase Yb genes are regulated is not well understood. The work described in this text is aimed at addressing the question of how the Yb_1 gene is regulated. A necessary step towards understanding regulation is isolation of structural gene. In this work, a Yb_1 cDNA clone was utilized as a probe in order to isolate the Yb_1 structural gene from a rat liver genomic library. Characterization of the gene was accomplished via nucleotide sequence analysis and facilitated direct comparison of structural as well as regulatory components of the Yb_1 gene to those of other transferase genes. Isolation and characterization of the Yb_1 gene also facilitated the initiation of a study aimed at identifying key cis- and trans-acting regulatory elements which are responsible for the elevation of gene activity in response to xenobiotic administration.

Historical Perspective

Eric Boyland was the first to recognize the significance of detoxification of xenobiotics by glutathione conjugation. In 1936, Boyland and Levi showed that a mercapturic acid was among the urinary metabolites of anthracene (3). At that time, the origin of mercapturic acids

was not known. Two decades later (1959), Boyland and others demonstrated that the acids were derived from glutathione conjugates (4-7).

The first demonstration of glutathione conjugation in vitro was the reaction of glutathione with the epoxide, 1,2-epoxy,1,2,3,4 tetrahydronaphthalene, to give S-(2-hydroxy,1,2,3,4 tetrahydro-1-naphthyl)-glutathione (8). The corresponding mercapturic acid N-acetyl-S-2 hydroxy-1,2,3,4, tetrahydronaphthyl L-cysteine was recovered from the urine of rabbits administered the epoxide (9). An analogous glutathione conjugate and mercapturic acid were obtained as metabolites of naphthalene. S-(2-hydroxy-1,2 dihydronaphthyl)-cysteine was isolated from incubations of naphthalene with rat liver slices (10) and N-acetyl-S(2-hydroxy-1,2 dihydro-naphthyl) cysteine was isolated from rats dosed with naphthalene.

These findings provided evidence that these epoxides were electrophilic metabolites of polyaromatic hydrocarbons and ultimately led to the discovery of the glutathione S-transferases in 1960. Booth and coworkers were the first to report enzymic catalysis of glutathione conjugation by the rat-liver-soluble supernatant fraction (10). The following year, the group demonstrated glutathione transferase activity for a number of electrophiles, including 1,2-dichloro-4-nitrobenzene-benzyl chloride, bromoethane, ethyl methano-sulphonate, bromosulfophthalein, and 1,2-epoxy 1,2,3,4 tetrahydronaphthalene together with those derived from naphthalene and anthracene by microsomal activation (11). Another demonstration of transferase activity was made at about the same time by Combes and Stakelum, who demonstrated that rat liver cytosol catalyzed glutathione conjugation of bromosulfophthalein (12).

The first transferase purified was isolated as a binding protein and called ligandin by Ketterer et al. in 1967 (13) and Litwack et al. in 1971 (14). The enzyme was tested against 1,2

dichloro-4 nitrobenzene, a favored substrate for glutathione S-aryl chloride transferase, but was found to have negligible activity (15). Later that year, the identity of ligandin with glutathione S-transferase B was recognized by Jakoby and his colleagues in collaboration with Arias and his group (16).

Nomenclature

Originally, the glutathione S-transferases were defined in the traditional manner according to the type of substrate. Examples include glutathione-S-aryl chloride transferase (17), glutathione-S-epoxide transferase (18), and glutathione-S-alkene transferase (19). Subsequently, it became obvious that although a number of different transferases existed, certain substrates were utilized by more than one enzyme. It is now accepted that individual glutathione transferases cannot be defined according to the chemical nature of the substrate.

In 1973, Clark et al. discovered that chloro-2-4 dinitrobenzene was a substrate for all known glutathione transferases (20). Using this substrate, four transferases were isolated by Jakoby et al. from the rat-liver-soluble supernatant fraction by gradient elution from carboxy-methyl-cellulose (21). The transferases were identified as transferase AA, A, B, and C. For some time the nomenclature of Jakoby, which was based on the naming of enzymes alphabetically in reverse order of their elution from the anion exchanger, was utilized. However, in 1977 Bass et al. showed that three major classes of glutathione transferase subunits from rat liver had different apparent molecular weights on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (22). Bass called these subunits Ya, Yb, and Yc in order of increasing apparent molecular weight.

In 1982, Mannervik and Jensen devised a new nomenclature, wherein each subunit was named by a single letter of the alphabet (23). However, it soon became obvious that

variants of the transferases in rat were too numerous to be covered by the alphabet. Consequently, in 1983 during a workshop on the glutathione transferases, researchers agreed to implement a numerical system. In this system, every subunit receives its own number. The identification of each transferase is based on the number given to its two constituent subunits. Table 1 summarizes the nomenclature which is used to identify the transferases. For the purpose of this text, the nomenclature of Bass et al. will be utilized.

The multifunctional nature of glutathione S-transferase.

The glutathione S-transferases are capable of catalyzing a broad spectrum of reactions; they are associated with a number of activities in addition to glutathione conjugation. One is glutathione peroxidase activity towards organic peroxides, such as cumene hydroperoxides, t-butylhydroperoxide and free fatty acyl hydroperoxides (24,25). Another is catalysis of glutathione-dependent isomerization (26). Additional reactions catalyzed by the transferases are the catalytic reactions of glutathione with organic nitrates and thiocyanates which result in the release of HNO_2 and HCN, respectively. These substrates are used by the Ya, Yb_1, and Yb_2 subunits (27). A fifth glutathione transferase-catalyzed reaction is formation of S-acetyl glutathione from 4-nitrophenyl acetate by the Ya, Yb_1 and Yb_2 subunits (28). Examples of some reactions catalyzed by the glutathione S-transferases are in Fig. 1.

Another important characteristic of the glutathione transferases is non-enzymic. This is the binding activity (hence the name ligandin). The transferases have been shown to bind a number of molecules with lipophilic moieties such as the endogenous ligands haem and bilirubin. The Ya subunit is very important with respect to binding these endogenous ligands (29). This subunit also appears unique in its ability to react covalently with photo-activated

Table 1. GSH transferase nomenclature[++]

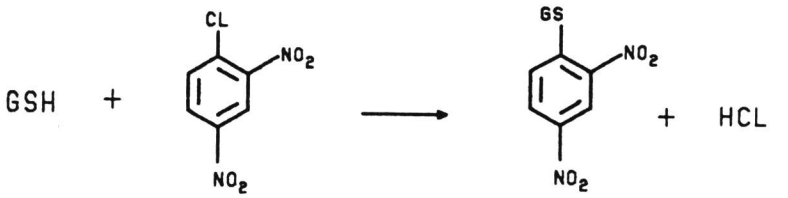
New Name	Previous Names			Mr*	pI+
1-1	B_1	YaYa	L_2	27000	9.1
1-2	B_2	YaYc	BL	--	9.1
2-2	AA	YcYc	B_2	30000	9.0
3-3	A	Yb_1Yb_1	A_2	28500	8.4
3-4	C	Yb_1Yb_2	AC	--	7.4
4-4	D	Yb_2Yb_2	C_2	28500	6.7
5-5	E	--	--	28500	7.0
6-6	--	YnYn	--	27800	5.3
7-7	--	YpYp	--	25500	6.8

[++]Data in this table taken from References 21, 22 and 23.

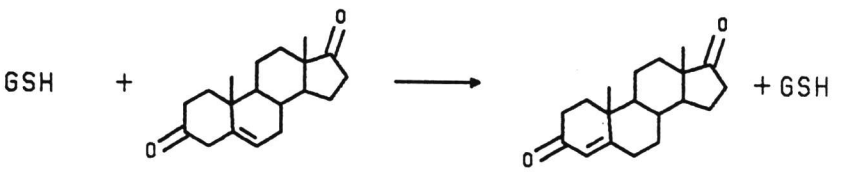
*Apparent molecular weight by sodium dodecyl sulphate polyacrylamide gel electrophoresis (using ovalbumin, carbonic anhydrase and soy bean trypsin inhibitor as standards).

+pI determined by isoelectric focusing.

(a) Conjugation



(b) Isomerization



(c) Peroxidation

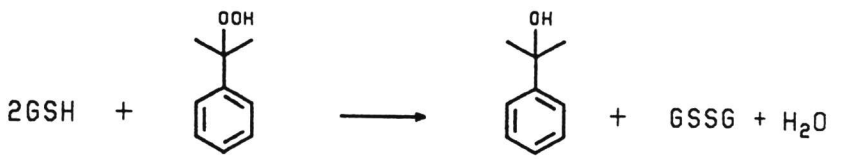


Fig. 1. Glutathione S-transferase Activities

bromosulfophthalein, metabolically activated benzo[a]pyrene, and amino azodye carcinogens (30).

The range of activity of the transferases is reflected by the names that have been given to these enzymes: namely, glutathione S-alkyltransferase, glutathione S-alkenetransferase, glutathione S-epoxide transferase, glutathione peroxidase, organic nitrate ester reductase, steroidisomerase, Δ^5-3-ketosteroid isomerase, ligandin, and phosphoric acid triester-glutathione alkyltransferase. With the exception of the steroid-designated enzymes and the peroxidase, all of these activities are now identified as glutathione S-transferase (EC 2.5.1.18.).

Purification

The purified rat liver glutathione S-transferases are homodimers or heterodimers comprised of at least eight subunits (Ya, Yb_1, Yb_2, Yc, Yd, Yp, Yk, Yn) which can be separated by one dimensional SDS-polyacrylamide gel electrophoresis (31-35). The proteins have molecular weights of about 50,000 daltons and are composed of two subunits of approximately equal molecular weights.

Most of the glutathione transferases in rat liver are proteins with an alkaline isoelectric point. Five transferases, AA, A, B, C, and E were purified to homogeneity by Jakoby et al. (35-38). These transferases are bound to carboxymethyl-cellulose as an early step in their purification and are named in the reverse order of their elution from the anion exchanger. Transferases A (38) and B (16) have been crystallized by Jakoby and coworkers. Transferase B is known to be highly organized with 40% α-helical and 15% beta-pleated sheet character (39).

Another transferase, designated M, is not bound by carboxymethyl-cellulose and was purified by Gillham (40) who noted that M binds to diethylamino ethyl-cellulose early in

purification. This transferase reacts with menaphthyl sulfate.

Askelof et al. have developed a purification method for transferases A and C which takes advantage of the basic nature of the rat liver transferases (41). More recently, Clark et al., have utilized affinity methods to isolate transferases in nearly pure form from the greater wax moth, Galleria mellorella (42). In their procedure, Clark et al., prepared an affinity column by coupling the thioether of glutathione and bromosulfophthalein to cyanogen bromide activated sepharose 4B. The transferases were eluted with either glutathione or with bromosulfophthalein and isolated in nearly pure form.

Isoelectric focusing has high resolving power and has proved useful in the separation of various isozymes of the glutathione S-transferases. Chromatofocusing has also been very useful in the separation of rat liver isozymes. Finally, the latest purification schemes have employed high performance liquid chromatography (HPLC and FPLC), thereby allowing protein separation in a time span of minutes.

The amino acid composition of transferases AA (39), A (37), B (37) and C (35) have been obtained by Jakoby et al. This data has lead to the conclusion that transferases A and C are very similar in gross composition whereas each of the others appear distinct. The specificity of antibodies produced separately against each of these transferases and against E are in agreement with this conclusion. Also, the cDNAs encoding the Ya and Yc subunits have been isolated and indicate that these subunits are similar.

The Human Transferases

Jakoby et al. have identified five glutathione S-transferases from human liver by elution from carboxymethyl-cellulose, and they are distinguished on the basis of their isoelectric points (43). All have been purified to homogeneity and are identified by Greek letters

($\alpha, \beta, \gamma, \delta, \epsilon$) in increasing order of isoelectric point. Transferase α has the lowest pI, while transferase ϵ is the most alkaline. All are dimeric proteins of approximately 49,000 daltons, composed of apparently identical subunits.

Assay Systems

The most convenient and sensitive assay for glutathione transferase activity is a spectrophotometric one in which glutathione and 1-chloro 2,4 dinitrobenzene serve as substrates (Fig. 2, Equation 1) (35).

Several spectrophotometric methods have been developed for a variety of compounds including an unsaturated ketone (trans-4-phenyl-3-buten-one) as substrate, (44), an epoxide [2-epoxy-3-(p-nitrophenoxy)propane] (36), and a sulfate ester (menaphthyl sulfate) (40). These reactions are depicted in Figure 2 (Equations 2-4, respectively).

Trimetric methods have also been developed for following the reaction of iodomethane (45) (Figure 2, equation 5). Also, several investigators have used radioactive substrates for assays of reactions with oxides (46,47), trisubstituted phosphates (48) and disulfides (49). Nitroglycerin as well as thiocyanates may each be used as substrates and may be followed by measurement of release of nitrite and cyanide, respectively (27).

Endogenous Substrates

Although most of the electrophilic substrates considered so far have been exogenous, there are several endogenous substrates for the glutathione transferases. One group of such substrates, the 4-hydroxy alkenals, are toxic products of fatty acid decomposition produced during lipid peroxidation (50,51). Another endogenous glutathione conjugate of physiological importance is the hormone leukotriene C_4 which is involved in local inflammatory reactions (52).

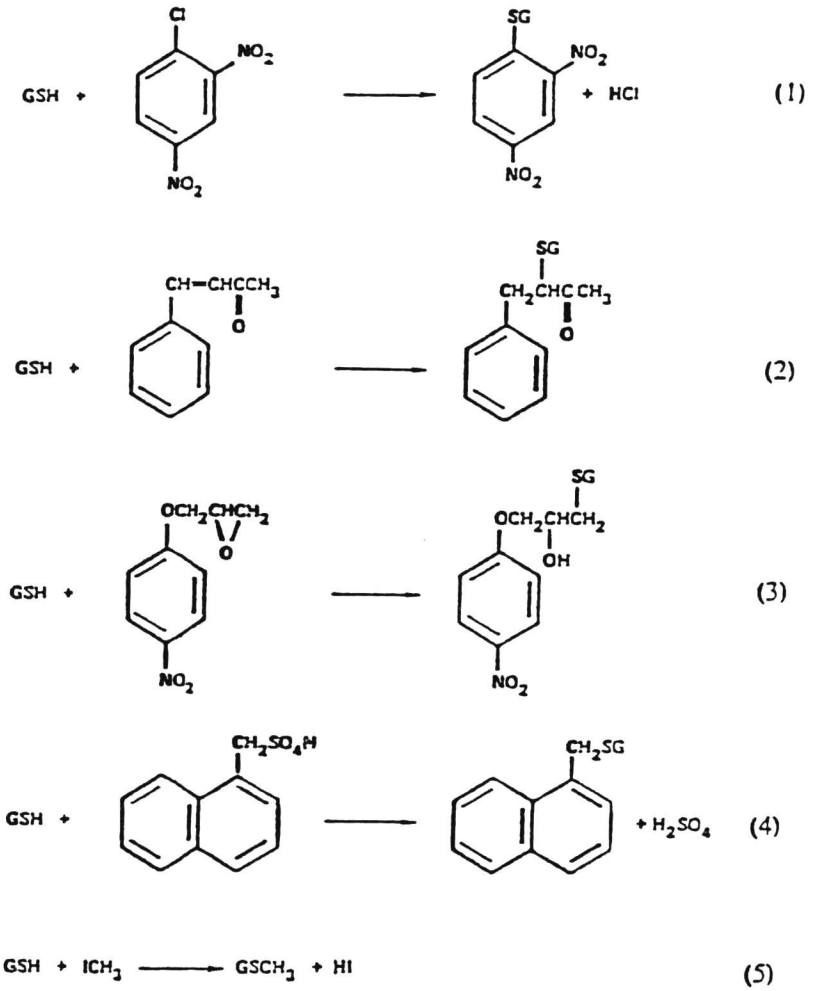


Fig. 2. Reactions Depicting Assays for Glutathione S-transferase Activity

Distribution of Rat Liver Glutathione S-Transferase.

1) Subcellular location and site of synthesis.

The early availability of antibody to glutathione S-transferase B allowed for quantitative estimates of availability of this isozyme, in normal rat liver, 45 μM/mg of soluble protein, in kidney, 22 μM/mg, and in small intestinal mucosa, 25 μM/mg (53). At the subcellular level, transferase B is also found in nuclei (54), microsomal membranes (55,56) and in intra-mitochondrial matrix space (57).

2) Tissue and species distribution.

In the rat, the liver is the most abundant source of the glutathione S-transferases. The transferases represent about 10% of the extractable protein of rat liver and can be induced to greater than 20% (55). Taking the rat as a whole, the Yb_2 subunit is the most abundant while the Ya is most abundant in liver and kidney.

Reports by several investigators suggest that the rat glutathione S-transferases are expressed in a tissue-specific manner. In 1979, Guthenberg and Mannervik isolated a transferase from rat lung that was not present in liver (58). A couple of years later, Scully and Mantle reported that the Ya subunit was absent from testis and that Yb subunit was absent from kidney (59). Tu et al. used in vitro translation and protein purification tools to study the differences in expression of the rat glutathione S-transferases in a number of tissues. Poly(A^+)-RNA was isolated from six rat tissues (kidney, lung, liver, heart, spleen and testis) and translated in vitro using the rabbit reticulocyte lysate system in the presence of ^{35}S-methionine. The glutathione S-transferase subunits synthesized in vitro were purified from translation mixtures by affinity chromatography on S-hexyl glutathione-linked sepharose

6B columns. The affinity bound fractions were analyzed by SDS-polyacrylamide gel electrophoresis. Using this methodology, Tu et al. identified a transferase subunit (M_r = 22,000) that was present in heart, kidney, lung, spleen, and testis but was lacking in liver (60).

Pemble et al. (61) have demonstrated that expression of glutathione S-transferase P occurs in a tissue-specific manner. In effect, mRNA hybridizing to the glutathione S-transferase P cDNA probe was detected in epididymis, kidney, adrenal gland, lung, spleen, skeletal muscle and heart but not in testis or normal liver.

Other supporting evidence for tissue-specific expression of the glutathione S-transferases comes from reports by Li et al. (62) and more recently by Abramovitz and Listowsky (63). Li et al. showed that the Ya subunit is not expressed in rat brain and demonstrated that two distinct transferase subunits (M_r=26,300 and M_r 25,000) were expressed in brain. Abramovitz and Listowsky isolated and characterized a novel Yb cDNA (Yb3) clone from a rat brain λ gt11 expression library and observed that the mRNA was 1300 bp in length and was expressed mainly in brain. This data was the first direct evidence that the Yb genes are expressed in a tissue-specific manner.

Finally, Hayes and Mantle employed Western blot analysis using polyclonal antibody to examine the tissue distribution of various glutathione S-transferases, namely the Yp, Yk, Ya, Yn, Yb and Yc subunits (64). Their data provides additional support at the protein level for tissue-specific expression of the glutathione S-transferase subunits.

The glutathione S-transferases are found in a wide variety of species including marine species such as elasmobranch, teleosts, and crustaceans (65), as well as in lower mammalian species, including ticks, flies, and locusts (17). The transferases are also found in higher mammals, such as monkey, guinea pig, and rabbit (66,67). Transferase activity is not

restricted to the animal kingdom; algae (68), higher plants (68), and certain bacteria (69), also have transferase activity.

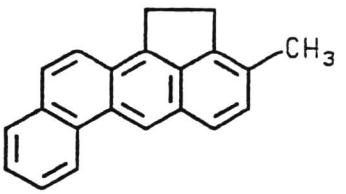
Inducers

The concentration of glutathione transferases in tissues is subject to regulation at several levels. Induction of the transferases has been reported following administration of phenobarbital (66), tetrachlorodibenzo-p-dioxin (TCDD) (70,71), methadone (72), and various pesticides (73-75) to experimental animals. The common antioxidant, BHA [2(3)-tert-butyl-4-hydroxyanisole], which is used by food processors, is an inducer of the transferases in mice and rats (76). Figure 3 contains the chemical structures of some known inducers of the transferases.

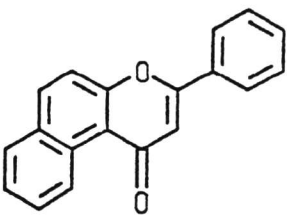
The Role of Glutathione S Transferase in Detoxification of Xenobiotics

The glutathione transferases have been intensively studied because of their role in the biotransformation of xenobiotics. The transferases are multifunctional in detoxification. They are important in the catalysis of reactions in which glutathione acts as a nucleophile; they serve as binding proteins, storing bilirubin in the liver, and they are capable of forming covalent linkages with alkylating agents.

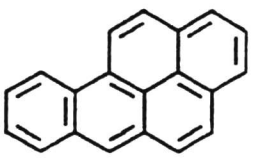
Figure 4 depicts a flow scheme of a possible biotransformation of an electrophilic species after conjugation with glutathione. The reactive electrophile may be any compound capable of forming a stable conjugate with glutathione. The X represents both the chemical groups that are eliminated (e.g. halogenides) and those that are modified without elimination (e.g. epoxides and alkenes). In the reaction catalyzed by the transferases, the S atom of glutathione (a) provides electrons for nucleophilic attack on the second electrophilic substrate (b). The newly formed glutathione conjugate (c) may then be excreted as such or hydrolyzed



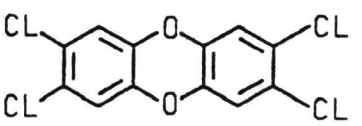
3-Methylcholanthrene



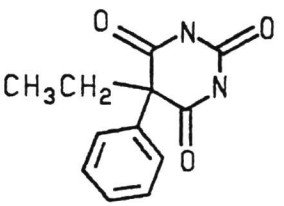
β - Naphthoflavone



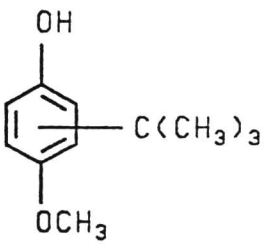
Benzo(a)pyrene



2,3,7,8-Tetrachlorodibenzo-p-dioxin



Phenobarbital



Butylated Hydroxyanisole

Fig. 3. Inducers of Glutathione S-transferases

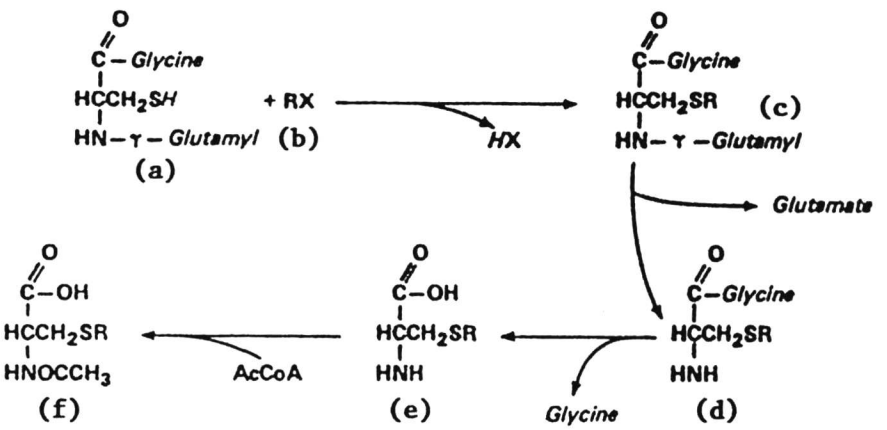


Fig. 4. Metabolic Pathway for the Synthesis of the Mercapturic Acids. The initial step is catalyzed by the glutathione S-transferases.

in two steps to an S-(substituent)-cysteine derivative (e). The cysteine conjugate can then be N-acetylated with acetyl Co enzyme A to yield a mercapturic acid or mercapturate (f), which is a classical urinary excretion product of xenobiotics (77).

Rat Liver Glutathione S-Transferase cDNAs.

Recent work on the glutathione S-transferases has focused on elucidating the mechanism by which the isozymes are regulated in response to xenobiotic administration. Much of the remainder of this text will focus on how molecular biology approaches have been used to unravel the complexity of the glutathione S-transferase subunits and their expression. Many of the studies which will be discussed will focus on the rat enzymes, since they are the most studied and are the focus of the work of the author.

Several labs have isolated cDNA clones complementary to the Ya, Yb_1, Yb_2 and Yc mRNAs (78-86). DNA sequence analysis of the clones has revealed an overall 66% nucleotide sequence identity between the Ya and Yc mRNAs and a 75% identity in nucleotide sequence in the protein coding region of these two mRNAs. Therefore, the Ya and Yc mRNAs and their corresponding proteins belong to one glutathione S-transferase gene family. The Ya subunit consists of 222 amino acids with a molecular weight of 25,547, and the Yc subunit is comprised of 221 amino acids with a molecular weight of 25,322. The sequence data has also revealed that more than one Ya gene is expressed in rat liver. To date, three full-length or nearly full-length Ya cDNA clones have been isolated and characterized. Two of the clones pGTR261 (79) and pGTB38 (80) differ by 15 nucleotides which translates into eight amino acid differences. The 3' untranslated regions of the two cDNAs are divergent. A third clone, pGTB45, is more similar to pGTR261 than to pGTB38, thus supporting the idea that the two clones are derived from distinct mRNAs encoded by separate genes (86). This clone, pGTB45,

contains a type 2 Alu repetitive element in the 3' untranslated region. The element consists of two overlapping polyadenylation signals downstream from the polyadenylation signal in pGTR261. The function of this element is unknown.

DNA sequence analysis of the Yb_1 and Yb_2 cDNA clones revealed that there is an 84% nucleotide sequence identity over the protein coding region of the two mRNAs (87-90). In the 3' untranslated region, there is only 32% sequence identity. The amino acid sequences of the Yb_1 and Yb_2 subunits share an 80% sequence identity. The Yb_1 polypeptide consists of 218 amino acids with a molecular weight of 25,919; the Yb_2 subunit is also comprised of 218 amino acids but has a molecular weight of 25,705. The amino acid sequence of the Yb_2 subunit has also been determined directly by conventional protein-sequencing procedures (91). The sequence of the purified protein agrees with that predicted from nucleotide sequence analysis of cDNA clones. Lai et al. have published the sequence of a second Yb_1 cDNA clone which differs from pGTA/C44 by four nucleotides in the coding region resulting in two amino acid differences (89). The 5' and 3' untranslated regions are identical to those of pGTA/C44.

More recently, Lai et al. (92) have reported the characterization of a Yb_4 cDNA clone which differs from other Yb cDNA clones by 40 to 53 amino acids. The Yb_4 cDNA clone shares significant homology (40 amino acid substitutions) to the Yb_1 cDNA clone pGTR200. There is 70% homology between the two 3' noncoding sequences. These data confirmed the existence of a second distinct glutathione S-transferase gene gamily.

Also noteworthy at this point is a report by Abramovitz and Listowsky on the characterization of a Yb_3 cDNA isolated from a rat brain λ gt11 expression vector library (63). This Yb_3 was shown to be a major form of transferase in rat brain while existing as a minor

form in the liver. The cDNA clone has greater than 80% sequence homology with the Yb_1 and Yb_2 cDNA clones. The amino acid sequence derived for the Yb_3 subunit has 28 amino acid substitutions unique to the Yb_3. The 3' untranslated region is totally divergent from all other isolated transferases.

Suguoka et al. (93) have reported the isolation of a glutathione S-transferase p cDNA from a library prepared from poly(A^+)-RNA isolated from 2-acetylamino fluorene-induced rat hepatocellular carcinoma. The cDNA encoding the Yp subunit revealed that the Yp polypeptide is comprised of 210 amino acids with a molecular weight of 23,307. The amino acid sequence of the Yp subunit was compared to that of the Ya and Yc subunits and was shown to possess an overall sequence identity of 32%, with homologous amino acids clustered in three regions of the amino acid sequence. Pemble et al. (61) have also isolated a cDNA clone, pGSTr7, which is identical to the Yp cDNA isolated by Suguoka et al. (93). This clone was isolated from a λ gt10 library that was constructed from N,N-dimethyl-4-aminoazobenzene-induced rat hepatoma.

Human Liver cDNA

Recently, Tu and Qian reported the isolation and nucleotide sequence analysis of a human glutathione S-transferase cDNA clone, pGTH1 which encodes a polypeptide of 222 amino acids (94). The nucleotide sequence of the coding region of pGTH1 is roughly 80% identical to the rat liver Ya and Yc clones and the deduced amino acid sequence has 75% identity to these same two subunits. The human cDNA sequence was found to be homologous to the rat liver Yb subunits near the NH_2 terminal region (amino acid residues 70-95). Interestingly, Tu and Qian found that residues 70-95 are conserved in all the glutathione S-transferase subunits.

Structural Analysis Liver Glutathione S-Transferase Genes

The availability of glutathione S-transferase cDNA clones has facilitated the isolation and characterization of the structural genes, which are essential in elucidating the mechanism by which the genes are regulated by xenobiotics.

1) The Ya-Yc gene family. In 1986, Telakowski-Hopkins et al. (95) isolated and characterized a rat liver glutathione S-transferase Ya gene. The Ya gene spans approximately 11 Kb and is comprised of seven exons separated by six introns. Exon 1 is 43 bp in length and encodes most of the 5' untranslated region of the Ya mRNA. Exon 2 is 109 bp in length and encodes amino acids 1-29 of the Ya subunit and 22 bp of the 5' untranslated region. Exon 3 is 52 bp and encodes amino acids 30-46 of the Ya polypeptide. Exons 4 and 5 are 133 bp and 142 bp in length and encode amino acids 47-91 and 92-139, respectively. Exon 6 is 132 bp in length and encodes amino acids 140-183 of the Ya polypeptide. Exon 7 is 234 bp in length and encodes amino acids 184-222 of the Ya protein and 121 bp of the 3' untranslated region of the Ya mRNA.

It is interesting to note that the amino acid sequence encoded by exon 3 represents the region that is most divergent between the Ya and Yc polypeptides. In this region there is only a 36% amino acid sequence identity between the Ya and Yc polypeptide, whereas there is an overall sequence identity of 66% between the two subunits. Exons 2 and 4 encode domains of the Ya subunit that are highly conserved in the Yc polypeptide. The amino acid residues of the Ya subunit encoded by exons 2 and 4 are 86% and 91% identical, respectively. The significance of conserved and divergent domains is not known; however, it has been suggested that the conserved exons encode domains of the subunits that impart similar functional properties such as glutathione binding site (95). Exons 3 and 5, which encode

divergent regions, are believed to encode domains of the subunits that are responsible for their substrate specificities. For example, the Ya subunit has high steroid isomerase activity, whereas the Yc subunit has high activity towards organic peroxides.

Finally, the precise number of Ya and Yc genes remains unknown. Southern blot analysis of genomic DNA using 5' and 3' regions of a Ya cDNA clone (pGTB38) indicates the presence of approximately five Ya genes and two Yc genes. It is unknown whether some of these genes are pseudo-genes or whether all of them are expressed in the liver. No laboratory has reported the characterization of a full-length glutathione S-transferase Yc structural gene.

A gene encoding the mouse Ya subunit has been isolated and characterized by Daniel et al. (96). The gene spans a distance of approximately 11 Kb, contains seven exons separated by six introns, and encodes an mRNA of 841 nucleotides. Promoter elements, namely TATA and CCAAT box sequences were located 32 and 70 nucleotides upstream from the initiation of transcription. The mouse Ya gene produces a 223 amino acid polypeptide that differs from the 220 amino acid rat Ya gene by 10 amino acid substitutions and a carboxyl terminus Phe-Lys-Ile-Gln instead of Phe-Lys-Phe.

2) The Yp gene family. The structural gene encoding glutathione S-transferase Yp subunit was isolated recently from a rat liver genomic library by Okuda et al. (97). The gene spans a distance of 3 Kb and like the Ya gene consists of seven exons separated by six introns. The start of transcription maps 70 nucleotides upstream from the translation initiation site.

Okuda et al. (97) have also determined the entire nucleotide sequence of a Yp genomic clone and have shown that it corresponds in sequence to the previously described cDNA clone, pGP5 (93). The group also identified several process-type pseudo genes,

which are believed to have originated by reverse transcription followed by insertion at specific sites.

3) The Yb gene family. Lai et al. have isolated and characterized a rat liver glutathione S-transferase Yb_2 gene (92). The gene spans a distance of 5 Kb and consists of eight exons separated by seven introns. The eight exons encode 12, 25 1/3, 21 2/3, 27, 34, 32, 37 and 29 amino acids, respectively. An analysis of this gene will be included later in this text under Discussion.

Regulation of Glutathione S-Transferases by Xenobiotics

Several studies have demonstrated that the glutathione S-transferases are elevated in the livers of rats administered 3-methylcholanthrene and phenobarbital. The increase in enzyme content and/or activity is paralleled by an elevation in the translational activity of the Ya, Yb_1 and Yb_2 mRNAs (98,99). Using in vitro translation systems and immunoprecipitation with Ya specific antibody, an increase in the translational activity of the Ya mRNA was detected 4 hrs after a single administration of phenobarbital. The induction was maximal (five to seven-fold) between 16 and 24 hr (100). RNA blot analysis using cDNA clones complementary to Ya mRNA confirmed that the increase in translational activity of this mRNA was due to an accumulation in the steady-state level of Ya specific mRNA (101). Using specific regions of the Yb_1 and Yb_2 cDNA clones in RNA slot blots, it was demonstrated that both of these mRNAs are also elevated by phenobarbital and 3-methylcholanthrene (101). Subsequently, the question arose as to whether the accumulation of glutathione S-transferase mRNAs in response to xenobiotics was due to transcriptional activation of the transferase genes. Nuclear run off assays were performed to answer this question. For the nuclear run off assays, nuclei were isolated from the livers of rats treated for various times with either phenobarbital or

3-methylcholanthrene (102). Using ^{32}P-UTP as the radiolabel, isolated nuclei were allowed to complete nascent RNA chains in vitro. ^{32}P-RNA was isolated and hybridized to various glutathione S-transferase cDNA clones. The transcriptional activity of the transferase genes was reflected by the amount of ^{32}P-RNA that hybridized. The transcriptional rate of the Ya genes was elevated five-fold at 8 hr after phenobarbital administration, while the Yb gene activity was elevated four fold at 6 hr after administration of the xenobiotic. With 3-methylcholanthrene, the transcriptional rate of the Ya genes was elevated eight-fold at 16 hr after administration of this xenobiotic while the activity of the Yb genes was elevated four-fold at 6 hr after administration. The extent of transcriptional activity was sufficient to account for elevation in mRNA levels in response to xenobiotics.

The mechanism by which the glutathione S-transferase genes are transcriptionally activated in response to xenobiotic administration is unclear. Recent work by Telakowski-Hopkins et al. (101) has centered on the construction of chimeric genes using 1.6 Kb of 5' flanking region of a glutathione S-transferase Ya gene fused to the structural gene encoding chloramphenicol acetyl transferase (CAT). The chimeric gene was transfected into rat, mouse, and human hepatoma cell lines and the 1.6 Kb Ya fragment directed CAT synthesis. When cells containing the Ya promoter fused to CAT were treated with β-naphthoflavone, a compound known to bind to the Ah (aryl hydrocarbon) receptor, CAT activity was elevated three to seven-fold in the hepatoma cell lines (101). When a chimeric gene containing CAT under the control of the SV40 promoter and enhancer was transfected into these cell lines, no increase in CAT activity occurred when β-naphthoflavone was added. This data suggested that the glutathione S-transferase Ya structural gene contained cis-acting regulatory elements that are responsible for transcriptional activation. The role of the 5' flanking region of the

glutathione S-transferase Ya subunit was studied further, and two cis-acting regulatory elements were identified. One regulatory element confers responsiveness to β-naphthoflavone, and the other regulatory element is necessary for maximum basal level expression. In addition to identifying two key regulatory elements, Telakowski-Hopkins et al. also demonstrated, using variant mouse cell lines defective in the dioxin receptor, that a functional Ah receptor is required for responsiveness of the regulatory element to β-naphthoflavone.

Sakai et al. (102) furthered their studies on the rat Yp gene by analyzing the cis-acting regulatory DNA elements of the gene. Various regions of the 5' flanking sequence of the gene were fused with the CAT gene and transcriptional activity was determined by the transient expression assay after introduction into a rat hepatoma cell line, dRLh84. Multiple regulatory elements were identified. Two enhancer elements were located 2.5 and 2.2 Kb upstream from the transcription start site. These elements were designated GPEI and GPEII. GPEI contains a consensus sequence for the phorbol ester (TPA) responsive element and GPEII contains two of the SV40 enhancer core-like sequences. A silencing element was also found 400 bp upstream of the cap site. The endogenous Yp gene as well as the exogenously translated gene were found to be stimulated when the rat fibroblast line 3Y1 was treated with phorbol 12-0-tetradecanoate 13-acetate (TPA).

The Role of the AH Receptor in the Regulation of Drug Metabolizing Enzymes.

In the cytochrome P-450 system, Whitlock and colleagues (103-105) have shown that the P_1-450 gene, the major 3MC inducible isozyme, contains a core promoter, dioxin responsive element, and a negative regulatory element that interacts with a repressor protein. Similar observations have been reported by Gonzalez and Nebert (106) and Fujisawa-Sehara et al. (107). Dioxin responsive elements (DREs) have also been identified upstream in the rat

(p450c) and human (p450) genes that correspond to the mouse P_1-450 by Sogawa et al. (108). Sogawa et al. (108) have deduced a consensus sequence 5' G N T A G C T C G G G G G 3' which is believed to form part of the dioxin-responsive element. Whitlock and colleagues have also devised a model to account for the regulation of the P_1-450 gene by polycyclic aromatic hydrocarbons (105). In the model, the AH or dioxin receptor binds polycyclic aromatic hydrocarbons, forming a receptor ligand complex that is translocated to the nucleus and interacts with a positive regulatory element. This interaction results in the transcriptional activation of the P_1-450 gene. Currently, it is unclear whether the AH receptor is directly or indirectly involved in the induction of glutathione transferase genes. Studies on the regulation of the transferases are aimed at determining whether this model accounts for transcriptional activation of these genes.

METHODS

Plating and Transferring of Genomic Library

One million plaques from a rat liver HaeIII genomic library cloned into λ Charon 4A were screened using a modification of the Benton and Davis procedure (109). The library was titered using serial dilutions and plated at a density appropriate for screening. The library was diluted to 2000 pfu/μl in SM (50 mM Tris pH 7.5, 100 mM NaCl, 13 mM $MgSO_4$, plus 2% gelatin). The plating bacteria were prepared by inoculating a single colony of E. coli LE392 into 50 ml of NZCYM (1% NZ amine, 0.1% casamino acids, 0.5% bacto-yeast extract, 85 mM NaCl, 13 mM $MgSO_4$) media supplemented with DT (0.2% maltose 0.1% diaminopimelic acid, and 0.04% thymidine) and incubating at 37° C overnight with shaking. The bacteria were collected by centrifugation at 4000 rpm for 10 min at room temperature. The pellet was then resuspended in sterile 0.01 M $MgSO_4$ [(0.4 x volume of original culture) or A_{600} = 2].

An aliquot (25 μl) of the library dilution (containing 2000 pfu/μl) was mixed with 0.25 ml LE392 plating bacteria and incubated at 37° C for 20 min. Following incubation 6.5 ml molten (55° C) NZCYM+DT top agarose was added, mixed by inverting, and poured onto prewarmed 15 cm NZCYM+ bottom agar plates. (Top agarose is 0.7% agarose; bottom agar is 1.5% agar)

The plates were incubated at 37° C for 15 hr or until minute plaques were visible. Plates were then chilled at 4° C for 4 hr in preparation for plaque lifts. Nylon filters were gently placed on the plates and the plates and filters were keyed with India ink. After 2 min the filters were gently pealed from the agarose, placed in 0.5 M NaOH for 3 min, and then placed on dry Whatman paper for 2 min. The NaOH treatment was repeated and the filters were again

placed on dry Whatman paper for 2 min. The filters were then neutralized in 1 M Tris-HCl, pH 7.5 for 5 min and allowed to air dry.

Plaque Hybridization

The glutathione S-transferase Yb_1 cDNA clone, pGTA/C44, was utilized to prepare a ^{32}P-labeled probe to screen the plaques. The ^{32}P labeled probe was prepared by nick translation of the plasmid DNA according to the method of Rigby et al. (101). To one microgram of plasmid pGTA/C44 was added 0.2 mM αdATP, 0.2 mM αdGTP, 0.2 mM αdTTP, 500 mM Tris-HCl (pH 7.8), 50 mM MgCl2, 100 mM β mercaptoethanol, 100 μg/ml nuclease free bovine serum albumin, and 200 μCi of $\alpha^{32}P$ dCTP. The reaction was initiated by the addition of 100 U of DNA polymerase I/DNase I and was incubated at 15° C for 60 min. The reaction was stopped by the addition of EDTA to 30 mM. The labeled DNA was separated from the nucleotides by subjecting the reaction mixture to gel filtration on an Sephadex G-75 (or ACA-54) column which was equilibrated with 10 mM Tris-HCl, pH 8.0/2 mM EDTA. Fractions (0.5 ml) were collected and those containing the peak of radioactivity were pooled and utilized for the hybridization.

The nylon filters, containing the fixed DNA, were pre-hybridized at 42° C for 6 hrs in 50% deionized formamide, 10% SDS, 1 M NaCl, 10% dextran sulphate, and 100 μg/ml salmon sperm DNA. Following pre-hybridization, a total of 4×10^5 cpm/ml pre-hybridization mix of pre-boiled ^{32}P-cDNA probe was added, and the filters were incubated overnight at 42° C. The filters were then washed in 2 x SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7.0.) at room temperature for 15 min, in 2 x SSC/1% SDS at 65° C for 60 min, and in 0.1 x SSC at room temperature for 20 min. The filters were air dried and exposed for autoradiography.

Putative positive plaques were selected by aligning master plates to the autoradiogram. Plaques corresponding to the center of positive signals were picked using a

sterile pasteur pipette. Plugs were expelled into 1 ml SM containing a drop of chloroform. Putative positive plaques were subjected to 3 additional cycles of screening until a 100% positive response was achieved, indicating plaque purity. Plaque plugs were amplified by plate lysis and stored at 4° C in the presence of chloroform.

Large Scale Preparation of Phage DNA

Phage DNA was isolated from the positive plaques using a modification of the Yamamoto procedure for plate lysis (111). Phage were plated (at a density of 10^5 pfu/150 mM plate) as described previously under plating of the genomic library. Plates were incubated overnight at 37° C and then chilled for 2 hr at 4° C. Following addition of 12 ml SM per plate, the plates were placed at room temperature on a shaker for 6 hr. Lysates were collected and pooled. To the lysates, NaCl (15 g/250 ml) was added and dissolved by rotating samples for 30 min at room temperature. This was followed by addition of polyethylene glycol (25 g/250 ml lysate) and continuous rotation at room temperature for 30 min. Lysates were then incubated at 4° C and rotated overnight. The phage lysates were then subjected to centrifugation at 4000 rpm for 15 min at 4° C and the pellet resuspended in 2 ml SM. The precipitate was extracted with 2 ml $CHCl_3$ and spun for 20 min at 6000 rpm at 4° C. The supernatant was collected and the volume adjusted to 3 ml by adding 5.6 M CsCl. The phage lysate was layered onto a CsCl step gradient containing 3.6 M, 4.0 M, and 5.6 M CsCl. The gradient was centrifuged in an SW-40 rotor at 30,000 rpm for 2.5 hr at 20° C. The phage DNA band (bluish-purple) was extracted and mixed with 0.1 volume of 200 x TE in a siliconized corning tube. One volume of deionized formamide was added and the mix was allowed to sit at room temperature for 30 min. One volume of H_2O was added, followed by addition of 6 volumes 100% EtOH. After mixing, phage DNA was precipitated for 5 min at room temperature and collected by centrifugation at 8000 rpm for 10 min. The pellet was

resuspended in 2 ml TE. The phage DNA was then treated with 100 μg RNase A, incubated at 37° C for 10 min, and treated with 20% SDS plus 400 μg proteinase K. The DNA was incubated at 65° C for 30 min, allowed to cool to room temperature, extracted twice with TE saturated phenol:$CHCl_3$:IAA (50:24:1), and extracted 3 x with TE-saturated ether. Phage DNA was precipitated by addition of 3 volumes of 100% EtOH and collected by centrifugation at 8000 rpm for 15 min at 20° C. The DNA pellet was dried under a gentle stream of N_2 and resuspended in TE.

Southern Blot Analysis

Fragments harboring sequence(s) complementary to regions of pGTA/C44 were identified by Southern Blot analysis (112). Genomic DNA was subjected to restriction digestion and fractionated on a 1% agarose gel in 1 x TAE (40 mM Tris-OH/20 mM acetic acid/2 mM EDTA) in the presence of 0.5 μg/ml ethidium bromide. The DNA was depurinated by incubating the agarose gel in 0.25 M HCl for 15 min at room temperature. Neutralization was achieved by incubating the gel in 1.5 M NaCl/0.5 M Tris-HCl, pH 7.5 for 30 min at room temperature with shaking. The gel was then placed on a wick pre-soaked (in SSC) in a gel electrophoresis unit. A nylon filter, pre-soaked in SSC, was placed over the gel, followed by addition of two pre-soaked Whatman 15 x 13 cm papers. Blotting paper was stacked over the Whatman paper and a 1 kg weight was placed atop this fixture. DNA was allowed to transfer region contains the element(s) overnight in SSC. Following transfer the filter was removed and immersed in 0.4 M NaOH for 60 sec to ensure complete denaturation of the immobilized DNA. The filter was then neutralized in 0.2 M Tris-HCl, pH 7.5/2 x SSC for 3 min and air dried.

The dried filter was subjected to prehybridization for 4 hr at 42° C in a mix containing 50% formamide, 1% SDS, 1 M NaCl, 10% dextran sulphate and 1 μg/ml salmon sperm DNA. DNA fragments containing the 5', middle and 3' regions of the glutathione S-transferase Yb_1

cDNA clone pGTA/C44 were utilized as radiolabeled probes as in the screening. A total of 4 x 10^6 cpm of radiolabeled probe was added and the hybridization was continued at 42° C overnight.

The filter was washed in 2 x SSC for 15 min at room temperature, in 2 x SSC/1% SDS for 30 min at 65° C, and in 0.1 x SSC at room temperature for 10 min. The filter was then air dried and exposed for autoradiography.

Isolation and Purification of DNA Restriction Fragments from Agarose Gels

Clones which harbored sequences complementary to the 5', middle, and 3' regions of pGTA/C44 were digested with the appropriate restriction enzyme (EcoRI) and the DNA was fractionated on a 1% agarose gel in TAE in the presence of 0.5 μg/ml ethidium bromide. The inserts of interest were visualized under UV light and excised using a razor blade. Gel slices were placed in microfuge tubes and the agarose dissolved by the addition of NaI and incubation at 37° C for 60 min with intermittant vortexing. Once the agarose was dissolved, glass agarose beads were added and the mix was incubated with rotation at 4° C overnight. Beads containing DNA were collected by centrifugation at 15,000 rpm 2 min at 4° C. The beads were rinsed 2 x in pre-chilled, recrystallized NaI and 2 x in 50% EtOH. Following centrifugation the beads were resuspended in 100 μl TE, and incubated at 37° C for 60 min. The supernatant was collected following centrifugation at 12,000 rpm for 5 min and stored on ice while the pellet was again resuspended in 100 μl TE and re-incubated at 37° C for 60 min. The supernatant was collected after centrifugation and pooled with the first elution. The DNA was precipitated by addition of 0.1 volume of 3 M NaOAc, pH 7.0 and 2 volumes of 100% EtOH and incubated on dry ice/EtOH for 15 min. The precipitate was spun at 15,000 rpm for 10 min at 4° C. The supernatant was removed and the pellet dried and resuspended in 60 μl TE. To

this was added 12 μl of 3 M Tris, pH 8.0 and 2 volumes of isopropanol. The DNA was reprecipitated on dry ice/EtOH 10 min, rinsed with 85% EtOH, dried, and resuspended in H_2O for short term storage or TE for long term storage.Subcloning of Genomic Fragments

The eluted Yb_1 genomic fragments were inserted into the plasmid vector pBr325. Chimeric plasmids were prepared by annealing 1 μg EcoRI digested dephosphorylated pBr325 to 0.5 μg of purified genomic fragment in the presence of 25 mM Tris-HCl (pH 7.8), 10 mM MgCl2, 4 mM ß-mercaptoethanol, and 0.4 mM ATP. One unit of T_4 DNA ligase was added and the reaction mixture was incubated at 4° C overnight.

Annealed chimeric plasmids were utilized to transform E. coli HB101 which were made competent by calcium chloride/heat shock treatment (113). Transformants were plated on L-agar plates containing 15 μg/ml tetracycline. Positive clones were identified as those clones which were viable on plates containing tetracycline but not viable on plates containing chloramphenicol.

Large Scale Plasmid Isolation

Colonies which were viable on L-tet plates but not viable on L-cam plates were sized by electrophoresis of a small quantity of EcoRI digested plasmid DNA from the colonies (114). The DNA was fractionated on a 1% agarose gel as described previously. Colonies whose inserts appeared to be of the expected size were grown on a large scale and the plasmid DNA was isolated. A single colony of the desired clone was inoculated into 100 ml L-broth containing 15 μg/ml tetracycline and incubated at 37° C overnight. The overnight culture was diluted 1:50 into M9 (18.7 mM NH_4Cl, 423 mM Na_2HPO_4, 21.7 mM KH_2PO_4, 85 mM NaCl, 2% casamino acids, 0.4 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.2% glucose, 10 mg/ml B1 containing

1 mg/ml uridine). The culture was incubated at 37° C until the optical density at 550 nM was 0.7-1.0. Spectinomycin (300 μg/ml) was added to the culture and incubation was continued overnight. Bacterial cells were collected by centrifugation at 5000 rpm for 10 min at 4° C, resuspended in 50 mM Tris-HCl, pH 8.0/25% sucrose and lysed by addition of 10 mg/ml lysozyme. The cells were placed on ice for 15 min with intermittent agitation, 0.25 M EDTA, pH 8.0 was added and the suspension was again incubated on ice for 5 min. Brij solution (1% Brij-58, 0.4% Na deoxycholate, 62.5 mM EDTA, 50 mM Tris-HCl, pH 8.0) was added and the suspension was again placed on ice for 15 min with occasional mixing. The cellular debris was pelleted at 35,000 rpm in a Beckman 50.2 rotor for 40 min at 4° C. Ethidium bromide (500 μg/ml) was added to the lysate and the refractive index was adjusted to 1.3940 with CsCl (0.8 g/ml). The lysate was cleaned by centrifugation at 12,000 rpm for 15 min at room temperature.

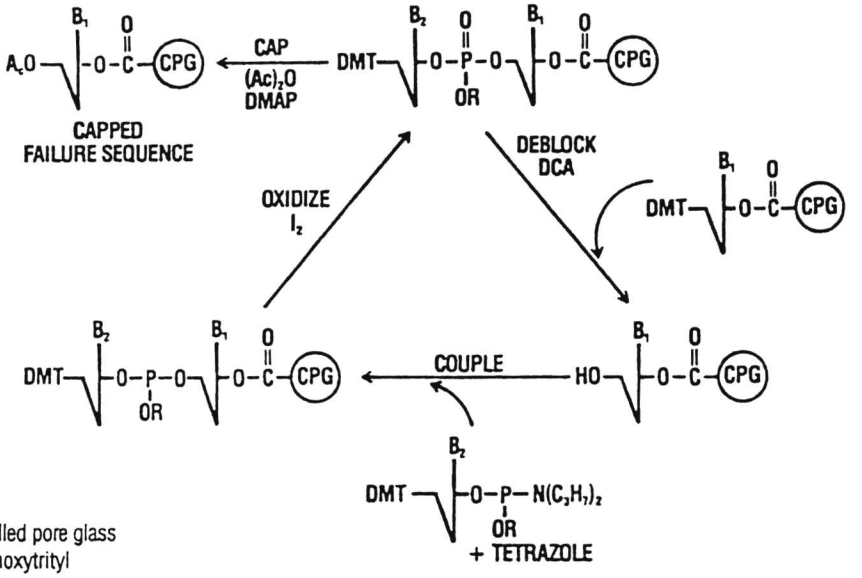
The DNA was isolated by centrifugation at 42,000 rpm in a Beckman 50.2 Ti rotor for 48 hr at 20° C. The band containing plasmid DNA was collected and the refractive index adjusted to 1.390 with CsCl. The DNA was again centrifuged at 42,000 rpm in a Beckman 70.1 Ti 48 hr at 20° C. The plasmid DNA was then collected, extracted 4 x with CsCl-saturated isopropanol, and dialyzed overnight in TE, pH 7.8. The plasmid DNA was extracted with an equal volume of phenol:$CHCl_3$:IAA (50:24:1). The aqueous phase was extracted 3 times with an equal volume of TE-saturated ether, and the DNA was precipitated at -20° C overnight by the addition of 0.1 volume of 3 M NaOAc, pH 7.0 and 2 volumes of 100% EtOH. The DNA was pelleted at 12,000 rpm in a Sorvall SS-34 for 45 min at 4° C, and resuspended in TE.

Oligonucleotide Synthesis and Purification

The oligonucleotides used in DNA sequence analysis were synthesized by standard cyanoethyl chemistry on a Biosearch Model 8600 DNA synthesizer (115). The DNA was synthesized in the 3'---5' direction with the first nucleotide bound to controlled pore glass in a prepacked reaction column.

The synthesis cycle included four major steps. First, the support bound 5' - DMT (dimethoxy trityl) group (located in a prepacked reaction column utilizing derivatized controlled pore glass as solid support) was removed with dichloroacetic, acid-generating a free 5'-OH for coupling. In the next step, the 5' OH was treated with a mixture of amidite and activator (1 H-tetrazole) resulting in the formation of a new phosphorus oxygen bond and thereby increasing the length of the oligonucleotide by one base unit. The third step was oxidation of the phosphorus (III) to phosphorus (V), a more stable oxidation state. This was necessary to prevent the dichloroacetic acid used in the deblocking from breaking the phosphorus (III) - oxygen bond. Finally, a capping procedure was utilized to block the untreated 5'-OH groups from any further reactions (See Figure 5).

At the conclusion of the synthesis, ammonium hydroxide was used to cleave the newly synthesized polynucleotide from the support and to remove the phosphoryl (cyanoethyl) protecting groups. The cleaved oligonucleotide was then incubated at 55° C for 5 hr to remove the protecting groups, dried under vacuum, and resuspended in 60% formamide, 10 μl xylene cyanol. The oligonucleotide was then heat denatured, loaded onto a 12% acrylamide/8 M urea gel, and electrophoresed until the xylene cyanol reached 12 cm. The gel was removed, overlayed onto Saran Wrap, and placed on a thin layer chromatography plate. The bands were visualized using ultraviolet light (short wave), excised using a razor blade and forced



CPG= controlled pore glass
DMT= dimethoxytrityl
B= base
R= $CNCH_2CH_2$-for beta cyanoethyl

Fig. 5. Amidite Synthesis Cycle

through a syringe in 5 ml elution buffer (0.5 M NH_4OAc/10 mM MgOAc, 1 mM EDTA). The gel slices in the elution buffer were placed on a shaker overnight at room temperature. The mix was then further purified by applying it to a Sep Pac column which was pre-equilibrated with 5 ml of 40% acetonitrile and washed with H_2O. Pressure was applied using a stream of N_2, the effluent was collected and re-applied to the column. The column was then washed 3 x with 5 ml H_2O and the DNA eluted with 4 ml 40% acetonitrile. The mix was lyophilized in a speed vac and resuspended with 1 ml H_20. The DNA was quantitated by reading the O.D. 260 nM. The concentration was adjusted to 10 ng/μl for use in sequencing.

Nucleotide Sequence Analysis

Plasmid DNA was further purified for sequence analysis by PEG precipitation. The DNA was incubated for 20 min on ice in NaCl plus 7.5% polyethylene glycol. The DNA was pelleted by centrifugation at 15,000 rpm for 15 min at 4° C. The pellet was rinsed with 70% EtOH, dried under vacuum, and resuspended in TE.

Supercoiled plasmid (2 μg) was resuspended in H_2O to 20 μl. To this mix, 2 μl of 2 M NaOH/2 mM EDTA was added and the mixture incubated at room temperature for 5 min. The mix was neutralized by the addition of 0.1 volume of 3 M NaOAc, pH 5.0 and the DNA was precipitated in 2.5 volumes of 100% EtOH. The pellet was rinsed with 70% EtOH, dried under vacuum and resuspended in H_2O.

For the sequencing reactions, two major systems were evaluated and modified for optimum results. The first was the Gem Seq K/RT sequencing system by Promega Biotec (Madison, Wisconsin) and the second was the Sequenase system by United States Biochemical (Cleveland, Ohio). For both systems, supercoiled DNA was purified, denatured, and neutralized as described above. Following neutralization, the purified denatured

template was annealed to the appropriate primer (synthetic oligonucleotide) and sequenced by standard dideoxy methodologies (116).

For the Gem Seq system, employing reverse transcriptase, the denatured template was resuspended in 34 mM Tris, pH 8.3, 50 mM NaCl, 0.5 mM $MgCl_2$, 5 mM DTT. (volume = 7 μl), mixed with 30 ng of primer (3 μl of 10 ng/μl stock), and incubated at 37° C for 1 hr. Chain elongation was accomplished by addition of 5 units of reverse transcriptase plus 4 μl of α^{32}P dATP (800 Ci/mmol) to the annealed primer/template. At this point, 3 μl of the label α^{32}PATP)/primer/template (mix was added to each of the four dideoxy nucleotide mixes and incubated at 42° C for 20 min. The formulation for the nucleotide mixes are listed in Table 2. Following incubation, 1 μl of chase solution (2 mM dATP, 2 mM dTTP, 2 mM dGTP, 2 mM dCTP in 34 mM Tris-HCl, pH 8.3, 50 mM NaCl, 6 mM $MgCl_2$, 5 mM DTT) was added to each mix and the 42° C incubation was continued for another 15 min. The reactions were stopped by adding 5 μl of the stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). The reaction mixtures were heat denatured at 90° C for 2 min, and loaded onto a prewarmed 6% acrylamide/8 M urea sequencing gel and subjected to electrophoresis in TBE (TBE = 89 mM Tris OH, 89 mM boric acid, 2 mM EDTA).

The Gem Seq system was also utilized with Klenow. The procedure is similar to the reverse transcriptase procedure with few modifications. For the annealing reactions, the Klenow buffer consisted of 10 mM Tris-HCl, pH 7.5, 50 mM $NaCl_2$. The formulations for the Klenow nucleotide mixes are in Table 3 and Table 4. For reactions including Klenow, the chain elongation was carried out at 37° C.

For the Sequenase system, the denatured template was annealed to 10 ng primer in sequenase buffer (40 M Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl) by first heating the

Table 2. Gem Seq K/RT System Reverse Transcriptase Nucleotide Mix Formulation*

Component	Nucleotide Mixes			
	G	A	T	C
ddGTP	50 μM	-	-	-
ddATP	-	3.6 μM	-	-
ddTTP	-	-	200 μM	-
ddCTP	-	-	-	100 μM
dGTP	250 μM	250 μM	250 μM	250 μM
dATP	-	-	-	-
dTTP	250 μM	250 μM	250 μM	250 μM
dCTP	250 μM	250 μM	250 μM	250 μM

*According to Promega Biotec Technical Manual, (Promega Biotech, Madison, WI) all nucleotide mixes contained 34 mM Tris-HCl pH 8.3, 6 mM $MgCl_2$, 50 mM NaCl, and 5 mM DTT.

Table 3. Gem Seq K/RT System Klenow Nucleotide Mix Formulations*

Component	Nucleotide Mixes			
	G	A	T	C
ddGTP	66 μM	-	-	-
ddATP	-	300 μM	-	-
ddTTP	-	-	117 μM	-
ddCTP	-	-	-	66 μM
dGTP	1.66 μM	33 μM	33 μM	
dATP	-	-	-	-
dTTP	33 μM	33 μM	1.66 μM	33 μM
dCTP	33 μM	33 μM	33 μM	1.66 μM

*According to Promega Biotec Technical Manual (Promega Biotech, Madison, WI) all nucleotide mixes contain 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT.

Table 4. Modified Klenow Nucleotide Mix Formulations*

Component	Nucleotide Mixes			
	G	A	T	C
ddGTP	100 μM	-	-	-
ddATP	-	20 μM	-	-
ddTTP	-	-	200 μM	-
ddCTP	-	-	-	100 μM
dGTP	5.4 μM	37.5 μM	54 μM	54 μM
dATP	-	-	-	-
dTTP	54 μM	37.5 μM	5.4 μM	54 μM
dCTP	54 μM	37.5 μM	54 μM	5.4 μM

*Nucleotide mixes contained 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT.

mix (total volume = 10 μl) 3 min at 65° C, then allowing the mixture to slow cool to below 30° C in a 65° C H_2O bath. To the annealed primer/template mix was added 10 mM DTT, 2 μl labeling mix (1.5 μM dGTP, 1.5 μM dTTP, 1.5 mM dCTP), 2.5 μl ^{32}P dATP (800 Ci/mmole) or ^{35}S-dATP (800 Ci/mmole), and 0.5 units of sequenase. The mixture was incubated at room temperature for 10 min and 3.5 μl of the label/primer/template mix was added to 3 μl of each dideoxy termination mix. The formulations for the termination mixes are in Table 5. The reactions were incubated at 37° C for 10 min and stopped by addition of 5 μl stop solution. The reaction mixtures were heat denatured and subjected to gel electrophoresis as described above in the Gem Seq procedure.

Isolation and Purification of Total RNA

Total RNA was isolated by the method of Chirgwin (117). Male Spraque-Dawley rats were injected intraperitoneally with 40 mg/kg body weight of 3MC. The rats were sacrificed at 16 hr post-injection and livers were obtained. The livers were washed in ice cold saline and all connective tissue was removed. The livers were minced using a sterile razor blade and homogenized in 4 M guanidine thiocyanate/0.5% sodium lauroyl sarcosine/0.025 M Na citrate/0. 1 M β-mercaptoethanol. Homogenates were centrifuged at 8,000 rpm for 10 min at 10° C in a Sorvall SS-34. The supernatant was decanted and the nucleic acids were precipitated at -20° C overnight by addition of 0.035 volume of 1 M acetic acid and 0.75 volume of 100% EtOH. The nucleic acid was pelleted as above and dissolved in 7.5 M guanidine hydrochloride/0.025 M Na citrate/5 mM DTT. The RNA was reprecipitated at -20° C overnight by addition of 0.025 volume of 1 M acetic acid and 0.5 volume of 100% EtOH. The RNA was pelleted and reprecipitated as above. The pellet was then rinsed with EtOH, dissolved in sterile H_2O and precipitated at -20° C overnight with 0.1 volume of 2 M KAc pH 5.0

Table 5. Nucleotide Mix Formulations for the Sequenase System*

	G	A	T	C
ddGTP	8 μM	-	-	-
ddATP	-	8 μM	-	-
ddTTP	-	-	8 μM	-
ddCTP	-	-	-	8 μM
dGTP	80 μM	80 μM	80 μM	80 μM
dATP	80 μM	80 μM	80 μM	80 μM
dTTP	80 μM	80 μM	80 μM	80 μM
dCTP	80 μM	80 μM	80 μM	80 μM

*According to Technical Bulletin, United States Biochemical, Cleveland, Ohio.

and 2 volumes 100% EtOH. The RNA was collected by centrifugation at 10,000 rpm for 30 min, dissolved in 5 ml sterile H_2O, and frozen at -70° C.

Oligo(dT)-Cellulose Chromatography

Poly(A^+)-RNA was isolated using affinity chromatography on oligo(dT) cellulose (118). Total RNA was heated at 68° C for 5 min and cooled to room temperature on ice. An equal volume of 20 mM Tris-HCl, pH 7.5/1% SDS/2 mM EDTA/1 M LiCl was added to the RNA and the mixture was applied to an oligo (dT)-cellulose affinity column which was equilibrated in 10 mM Tris, pH 7.5/0.5% SDS/1 mM EDTA/0.5 M LiCl. The column was rinsed with the same buffer. The poly(A^+)-RNAwas eluted with 10 mM Tris-HCl, pH 7.5. Fractions containing the RNA, as determined by absorbance at 260 nM, were pooled and the RNA was precipitated with 0.1 volume of 2 M KAc pH 5.0 and 2 volumes of 100 EtOH at -20° C overnight. The RNA was collected by centrifugation at 12,000 rpm and the pellet dissolved in sterile H_2O.

Primer Extension Analysis

The transcription start site was determined by primer extension analysis (119). An oligonucleotide primer (5' CCC-AGT-ATC-ATA-GGC-ATG-GTT-CTG-GCG-CTG-TGG 3'), which represents a 33 bp fragment complementary to the 5' region of the glutathione S-transferase Yb_1 mRNA was synthesized, purified as described previously, and labeled at the 5' end with γ^{32}PdATP. Briefly, 1500 ng of the oligonucleotide was mixed with kinase buffer, (10 mM $MgCl_2$ 5 mM DTT, 50 mM Tris HCl, pH 2.5, 0.05 mg/ml BSA), [γ^{32}P-ATP], plus 10 units of T_4 polynucleotide kinase, and the mixture was incubated at 37° C for 30 min. The kinase was inactivated by heating the reaction mix at 65° C for 5 min. The ^{32}P-labeled oligonucleotide was precipitated by addition of 0.1 volume of NH_4OAc plus 10 volumes of 100% EtOH, in a dry ice bath for 30 min. The DNA was pelleted by centrifugation for 15 min at 15,000 rpm,

resuspended in H_2O, and reprecipitated twice as above. The DNA was then resuspended in 0.3 M NaOAc and quantitated by scintillation counting.

A total of 5 x 10^4 counts of labeled oligo was added to 30 μg poly(A^+) RNA. The salt concentration was adjusted to 0.3 M NaOAc, 2.5 volumes of 100% EtOH added, and the mix precipitated as above. The DNA:RNA hybrid was dried by inverting on a Kim wipe for 30 min, resuspended in 30 μl hybridization buffer (80% deionized formamide, 40 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA) and incubated at 30° C overnight. The DNA:RNA hybrid was precipitated as above, spun at 15,000 rpm for 15 min, and rinsed with 75% EtOH/25% 0.1 M NaOAc, pH 5.3. The pellet was dried as above and resuspended in twenty-five microliters of reverse transcriptase (RT) buffer containing 50 mM Tris·HCl, pH 8.0, 5 mM $MgCl_2$, 5 mM DTT, 50 mM KCl, 0.5 mg/ml BSA 0.6 mM dNTPs, (dATP, dCTP, dTTP, dGTP), plus human placental ribonuclease inhibitor.

Reverse transcriptase (40 units) was added and the reaction mix incubated at 42° C for 2 hr. The reaction was stopped by addition of EDTA to 40 mM. Next, 50 μg ribonuclease A were added and the mix incubated at 37° C for 30 min. The mix was extracted with an equal volume of phenol:$CHCl_3$:IAA (50:24:1). The aqueous phase was transferred to a fresh tube, and the RNA was precipitated by addition of 3 volumes 100% EtOH, rinsed in 70% EtOH, and dried under vacuum. The pellet was resuspended in 3 μl TE plus 4 μl formamide loading buffer, boiled for 3 min, and quick cooled on ice. A 3 μl aliquot was run on a 12% polyacrylamide/8 M urea sequencing gel. A sequence mix of M13mp19 was run alongside the extended primer in order to provide a size marker.

DNA Amplification Using Polymerase Chain Reaction

A new technique which allows in vitro amplification of DNA, called the polymerase

chain reaction (PCR), was utilized for generating a series of Yb_1 promoter deletions (120,121). The amplified fragments were subsequently inserted in the reporter vector, pSV_OCAT, a plasmid vector, containing the structural gene encoding chloramphenicol acetyl transferase (141).

The success of the polymerase chain reaction is based on the thermal stability of Taq polymerase, an enzyme isolated from Thermus aquaticus. PCR amplification involves the use of two oligonucleotide primers that flank the DNA segment which will be amplified, and repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers with the Taq polymerase. The primers hybridize to opposite strands of the target sequence and are oriented so that DNA synthesis by the polymerase extends across the region between the primers (see Figure 6). Since the extension products are complementary to and capable of binding to the primers, each successive cycle doubles the amount of DNA synthesized in the previous cycle. The net result is exponential accumulation of the target fragment at a rate of 2^n, where n is the number of cycles.

To generate the DNA fragments containing the Yb_1 promoter region, the clone containing the 1.9 Kb EcoRI/BglII fragment representing the Yb_1 5' flanking region, was utilized as the template for all amplifications. The oligonucleotides were designed with a Hind III restriction site on the 5' end to facilitate cohesive-end ligations. It was determined that an additional 4-5 bases on the 5' end were necessary for successful restriction enzyme digestion of amplified fragments. The additional nucleotides chosen at random were TGACG.

For the reactions, the template (15 ng) was mixed with 1 μg of each of the designated oligonucleotides (5' end and 3' end), 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP and 0.2 mM

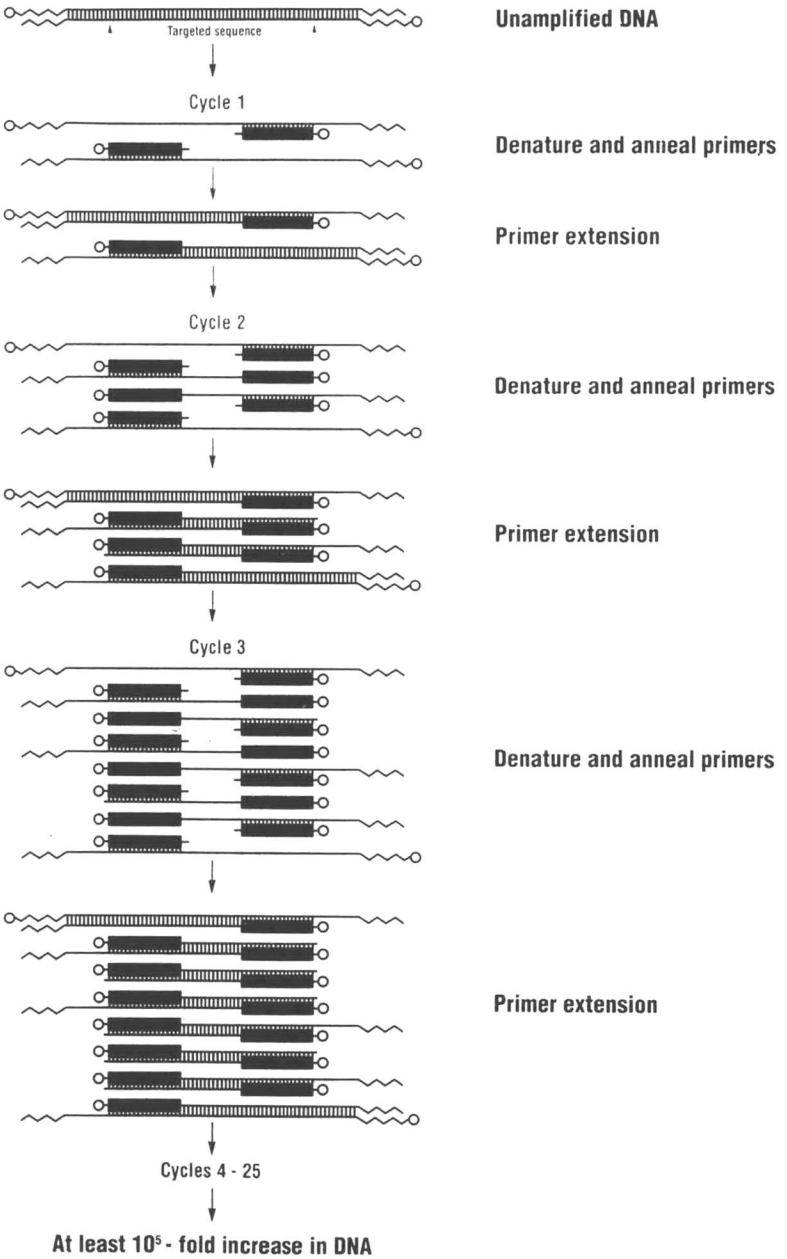


Fig. 6. Polymerase Chain Reaction

dCTP, in presence of reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin). The mixture was overlayed with 100 μl sterile mineral oil to prevent sample evaporation, and heated for 3 min at 94° C to inactivate nucleases and proteases. After quick cooling one minute on ice, 0.5 units of Taq polymerase was added and the cycle of incubations initiated using a preprogrammed Zymark model MRK-15 robot with 3 heating blocks. The PCR cycle consisted of 3 incubations: 2 min at 94° C for denaturation, 2 min at 58° C for annealing (oligo to template), and X min at 72° C for elongation. The value for X was determined by dividing the total length of the DNA to be amplified (base pairs) by 200 (the number of bps/min polymerase extends) and adding an additional minute to ensure optimum elongation. The reaction efficiency was monitored by subjecting an aliquot of the product to agarose gel electrophoresis. A total of 12 different fragments were generated using the PCR technique ranging in size from approximately 70 bps - 2000 bps.

Construction of Yb_1-pSVO CAT Chimeric Genes

One clone, pGTA 1.9 CAT was generated by inserting the EcoRI/BglII 2 Kb fragment into pSV_0CAT. This fragment hybridized to the 5' end of the Yb_1 cDNA clone pGTA/C44, and contained the putative promoter. The overhang generated by the enzymes was filled in using the Klenow fragment (122). The fragments were annealed using T_4 DNA ligase and transformed into E. coli HB101. Another clone, pGTA 1.9 CATB, was generated by digesting pGTA 1.9 CAT with SacII, subjecting the linearized clone to digestion with Bal-31 exonuclease, filling in overhangs with Klenow, and re-ligating the shortened clone in presence of T_4 ligase. Briefly, 10 μg linearized pGTA 1.9 CAT was incubated for one minute at 30° C in the presence of 20 mM Tris HCl pH 8.0, 0.6 M NaCl, 12.5 mM $MgCl_2$, 12.5 mM $CaCl_2$ and 3 units of Bal-31 nuclease "fast" form. The reaction was stopped by adding EDTA to 50 mM.

The amplified DNA fragments were subjected to restriction enzyme (HindIII) digestion, extracted with phenol:$CHCl_3$:IAA (50:24:1), precipitated in 0.1 volume of 3M NaOAc plus 2 volumes of 100% EtOH, rinsed with 70% EtOH, spun at 15,000 rpm for 15 min and the pellet dried under vacuum. The DNA pellet was resuspended in water, and approximately 0.5 μg of the purified fragment was mixed with 1 μg linearized (with HindIII), dephosphorylated pSV_OCAT. The fragment was annealed to the vector as described previously. The chimeric plasmid was then transformed into competent SCS1 cells (Stratagene). The transformation procedure was modified to yield approximately 1000 transformants. SCS1 cells were incubated in presence of β-mercaptoethanol for 10 min on ice. To this mix was added approximately 250 ng of chimeric plasmid. The mixture was incubated on ice for 30 min, heat shocked at 42° C for 1 min and cooled on ice for 2 min. The cells were allowed to propagate in the presence of 0.7 ml of S.O.C. (2% bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) media by incubating at 37° C with shaking (225 rpm) for 1.5 hrs, the transformants were plated on L-agar plates containing 100 μg/ml ampicillin.

Clones containing desired inserts were identified by fractionating a small quantity of Hind III digested plasmid DNA from the colonies using agarose gel electrophoresis as described previously. Clones containing inserts in the proper 5'---3' orientation were identified by double restriction enzyme digests in some cases, and nucleotide sequence analysis employing the Sequenase system in other cases. Clones harbouring inserts of interest were grown on a large scale and the plasmid DNA isolated as described previously. The clones were grown initially in the presence of ampicillin and were amplified in the presence of 250 μg/ml chloramphenicol.

Transfection of Yb_1 Promoter and Promoter Deletions into Mammalian Cells

Chimeric plasmids were introduced into a human hepatoma cell line (Hep G2) in order to study transient expression of the Yb_1 promoter-CAT chimeric genes. Hep G2 cells were grown in Eagle's minimum essential medium, nonessential amino acids, sodium pyruvate, 10% (vol/vol) fetal bovine serum, penicillin at 10 units/ml, and streptomycin at 10 units/ml at 37° C in a 7% CO_2 chamber. All cell culture work was performed in a sterile environment.

For the transfections, 10 μg of CsCl purified plasmid DNA was mixed with 240 μl of sterile H_2O and 31 μl of 2 M CaCl2 (123). This mixture was added to 250 μl of 2 x HBS, pH 7.12 and the precipitated DNA was added to a 25 cm^2 flask containing approximately 0.6 x 10^5 cells (70% confluence) in 4 ml enriched media. The cells were incubated at 37° C in a 5-7% CO_2 environment for 4-5 hr. The media was removed and the cells rinsed with PBS. Cells were then subjected to a glycerol shock by incubation at room temperature in the presence of 15% glycerol for 2 min. Cells were then rinsed with room temperature media followed by addition of 4-5 ml fresh media and incubation overnight at 37° C in 5-7% CO_2. After overnight recovery, cells were incubated in the presence of either 50 μM BNF, 50 mM 3MC, or 2 mM PB overnight, to determine the effect of the xenobiotics on transcriptional activity of the Yb_1 promoter and promoter variants.

Cells were harvested in a nonsterile environment. Briefly, the media was removed, the cells rinsed twice with room temperature PBS. The cells were removed from the side of the flask by treatment with 1 ml trypsin-EDTA for 2-3 min. The cells were rinsed off of the flask with 4 ml PBS, placed in a conical tube, and spun at 1000 rpm for 4 min. The supernatant was aspirated and the pellet was resuspended in 100 μl of 0.25 M Tris pH 7.5-7.8. The cells

were transferred to a 1.5 ml microfuge tube and lysed by three successive incubations in a dry ice/EtOH bath for 5 min then at 37° C for 5 min (with mixing between the 37° C and dry ice incubation). The cell lysate was collected by centrifugation at 15,000 rpm for 4 min. The supernatant was transferred to a new tube and the lysates stored at -20° C (short term) and -70° C (long term).

Protein Assay

The cell extracts were subjected to protein quantitation using a Biorad Protein Assay, a colorimetric assay for measuring total protein concentration. The assay is based on the color change of a coomassie blue G-250 dye in response to various concentrations of protein. The dye has an absorbance maximum at 595 nM and thus the A_{595} was recorded for all samples. A linear regression program was utilized to calculate the protein concentration of the cell extracts.

Chloramphenicol Acetyl Transferase Assay (CAT)

Protein concentrations were normalized for all CAT assays. From 5-50 μg of protein (depending on cell type) were incubated in 0.25 M Tris-HCl, pH 7.5 (to 160 μl), 0.5 μCi ^{14}C-chloramphenicol, and 20 μl of 4 mM acetyl CoA (124). The mixtures were incubated at 37° C for 60 min and placed on ice. The reaction mixtures were then extracted with 200 μl ethyl acetate. The upper aqueous phase was removed to a new 1.5 ml tube and the bottom layer re-extracted with 200 μl ethyl acetate. The aqueous phases were pooled, dried under vacuum, resuspended in 25-30 μl ethyl acetate and spotted onto a thin layer chromatography (TLC) plate. The TLC plate was placed in a tank which had been equilibrated with 100 ml chloroform:methanol (95:5) for 45 min - 1 hr. The plate was allowed to air dry, covered with Saran Wrap, and subjected to autoradiography.

RESULTS AND DISCUSSION

Isolation of a glutathione S-transferase Yb_1 gene

One million plaques from a rat liver HaeIII genomic library cloned into λ Charon 4A were plated and screened by *in situ* hybridization to a ^{32}P-cDNA probe. The probe was prepared by nick translation of the glutathione S-transferase cDNA clone, pGTA/C44. A partial restriction map of pGTA/C44 is shown in Fig. 7.

Screening of the plaques resulted in the isolation of six clones containing sequences complementary to glutathione S-transferase Yb_1 mRNA. Positive plaques were subjected to 3 additional rounds of screening in order to achieve plaque purity. A typical autoradiogram from each cycle of screening is represented in Fig. 8. Pure plaques were propagated and phage DNA isolated as described in Methods. The size of the insert(s) in each clone was determined by restriction digestion of the phage DNA with EcoRI. The digests were fractionated on a 1% agarose gel. The restriction digest of the clones is shown in Fig. 9.

Southern blot analysis was utilized to positively identify the putative Yb_1 gene. Genomic DNA was transferred to a nylon membrane and hybridized to ^{32}P-labeled fragments representing the 5', middle, and 3' regions of the Yb_1 mRNA. The fragments were generated by restriction digestion of pGTA/C44 with Pst1/Sph1. One clone, λ GTA/C22, harbored inserts which hybridized to all 3 regions of the Yb_1 mRNA and was selected for further characterization (Fig. 10).

Subcloning of Yb_1 genomic fragments

The glass bead purified EcoRI digested genomic fragments, approximately 2.5 Kb and 5 Kb in size, were annealed to EcoRI digested dephosphorylated pBr325 and used to transform *E. coli* HB101. Since the inserts were cloned into the site of chloramphenicol resistance, positive tranformants were identified as those which were tetracycline resistant

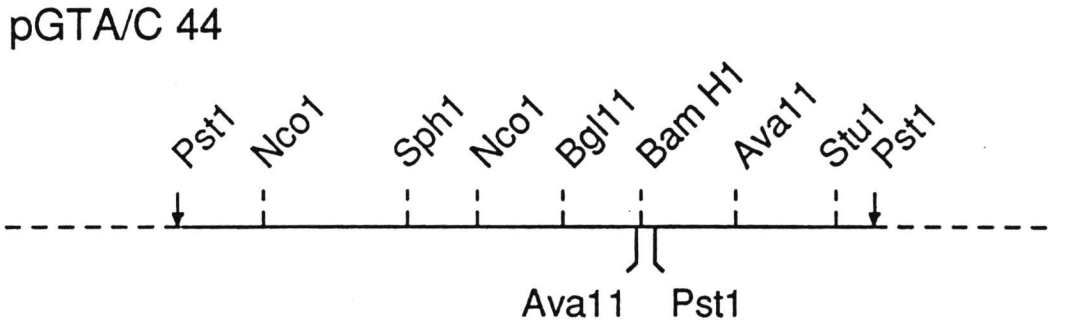


Fig. 7. Partial restriction map of pGTA/C44

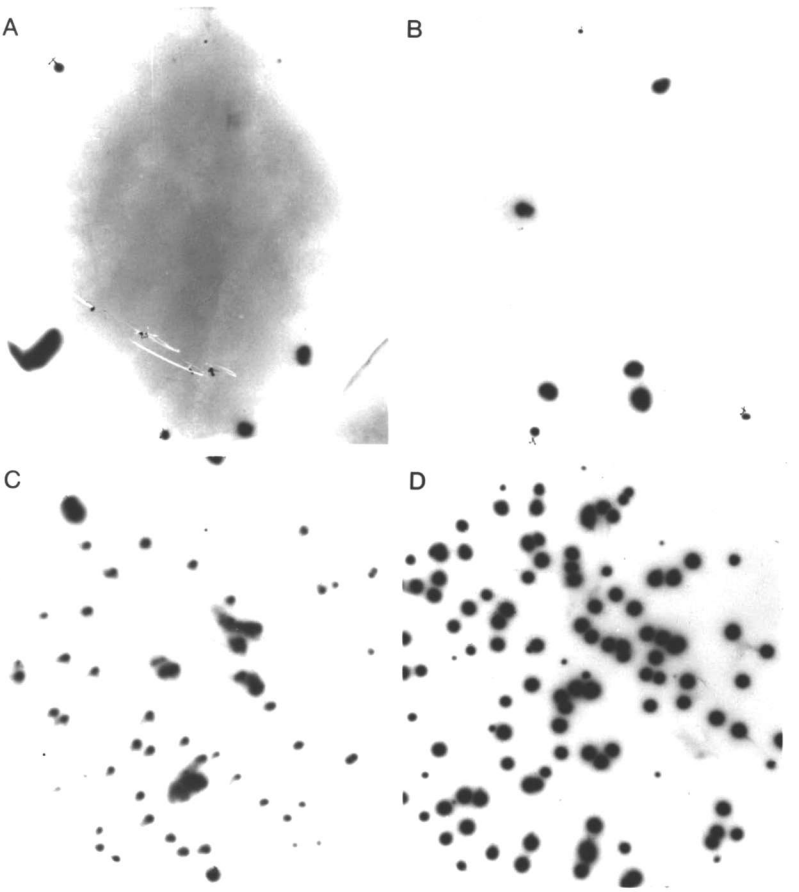


Fig. 8. Autoradiograph of in situ hybridization of plaques. Plaques were lysed in situ and the DNA was denatured and fixed to gene screen. The DNA was hybridized to a ^{32}P-cDNA probe (pGTA/C44) prepared by nick translation. Hybridizing plaques were subjected to 3 additional cycles of screening until plaque purity was achieved. Figure A depicts round 1, B - round 2, C - round 3, D - round 4.

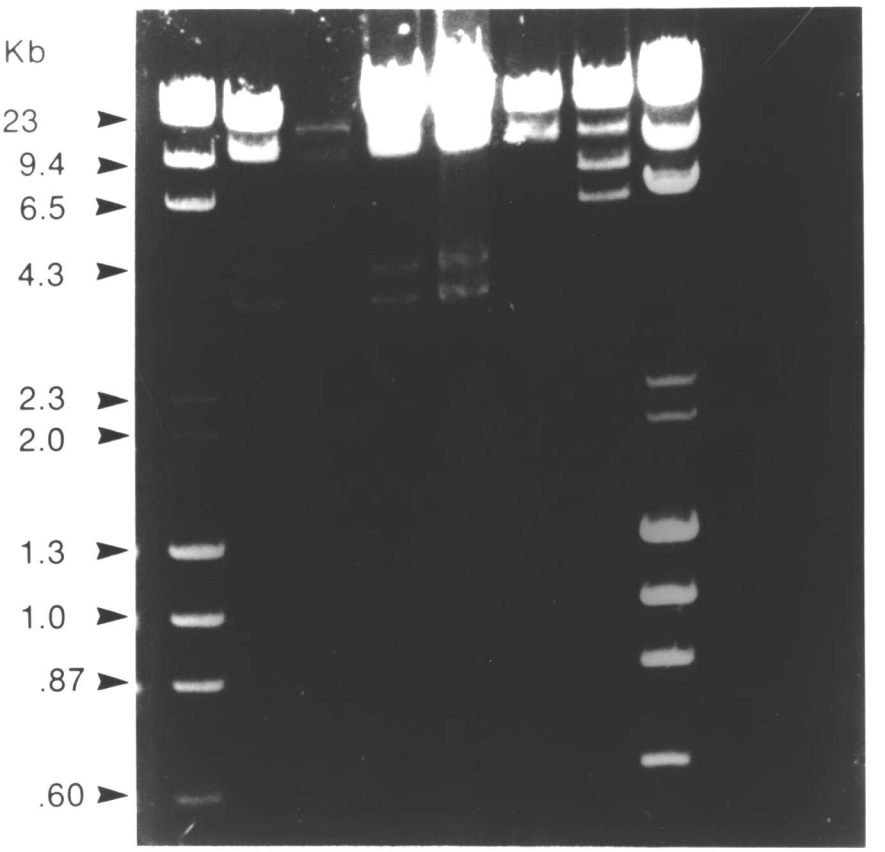
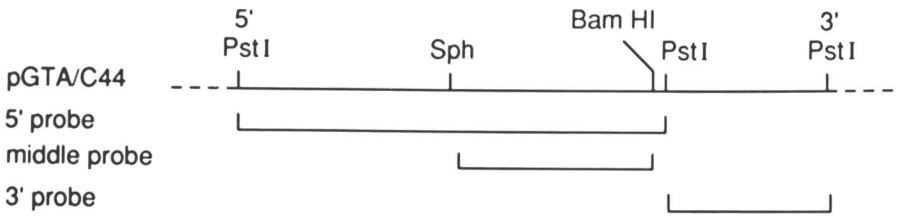


Fig. 9. EcoRI digestion of positive clones on 1% agarose stained with ethidium bromide. The clones were digested to completion with EcoRI and fractionated on a 1% agarose gel in the presence of 0.5 μg/ml ethidium bromide. Lanes 1 & 8 contain HindIII digested λ DNA o x 174 DNA fragments. Digestion of 5 clones yielded 4 bands: 19 Kb, 11 Kb, 4.4 Kb, 3 Kb (Lanes 2-6). Digestion of the 6th clone yielded 5 bands (Lane 7): 19 Kb, 11 Kb, 7 Kb, 6 Kb, and 2.5 Kb.



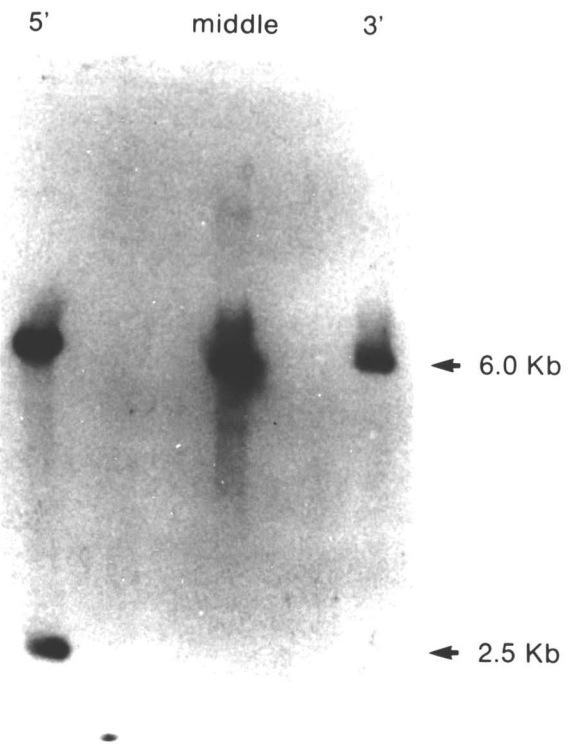


Fig. 10. Southern blot analysis of λ GTA/C22. DNA was digested with EcoRI, fractionated on 1% agarose in the presence of 0.5 μg/ml ethidium bromide, transferred to nylon discs and subjected to hybridization to ^{32}P-DNA fragments obtained by Pst1/Sph1 digestion of pGTA/C44. The PstI/SphI fragments represent the 5', middle, and 3' regions of the glutathione S-transferase-Yb_1 mRNA.

(Tet^r) and chloramphenicol sensitive (Cam^s). Clones harboring inserts of the appropriate size were grown on a large scale and the plasmid DNA isolated.

Characterization of a glutathione S-transferase Yb_1 gene

The rat liver glutathione S-transferase Yb_1 structural gene is contained within two EcoRI fragments. The two genomic clones, pGTA 5 K and pGTA 2.5 K, were subjected to DNA sequence analysis using a novel approach involving the use of double stranded plasmid DNA template and synthetic oligonucleotide. Oligonucleotides were synthesized on a Biosearch Model 8600 DNA synthesizer employing beta-cyanoethyl, solid phase chemistry. Three different sequencing systems were evaluated and in some cases modified for use in DNA sequence analysis. The sequencing reactions were carried out as described in Methods. Briefly, purified ds DNA was denatured in alkali, annealed to the desired oligonucleotide primer, and the primer extended in the presence of one labeled dNTP and the other three cold dNTPs.

The exon-intron structure of the Yb_1 gene is represented in Fig. 11. The gene spans a distance of approximately 5.5 Kb and consists of eight exons separated by seven introns. The nucleotide sequence of each exon and all exon-intron junctions are presented in Fig. 12A and Fig. 12B.

Exon 1 is 73 bp in length and encodes the 5'-untranslated region of the Yb_1 mRNA from nucleotides 1-37 as well as amino acids 1-12 of the Yb_1 polypeptide. Exon 2 is 76 bp in length and encodes amino acids 13-37 of the Yb_1 subunit. Exons 3 and 4 are 65 bp and 82 bp in length and encode amino acids 38-59 and 60-86, respectively. Exons 5 and 6 are 101 and 96 bp in length and encode amino acids 87-120 and 121-152, respectively. Exon 7 is 111 bp in length and encodes amino acids 153-189 of the Yb_1 protein. Finally, exon 8 is 458 bp in length

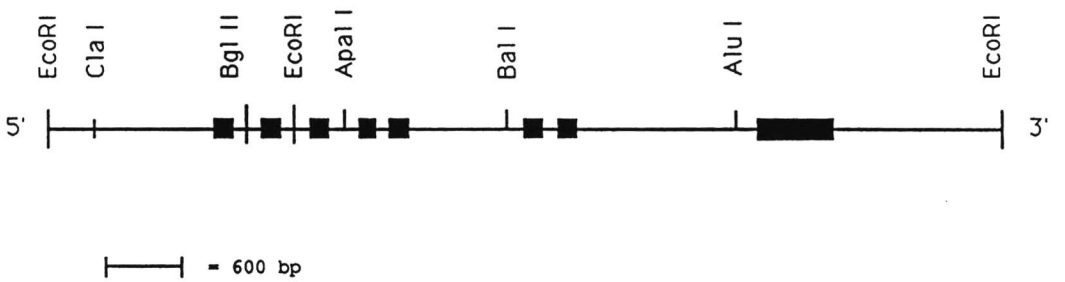


Fig. 11. The exon-intron structure of glutathione S-transferase-Yb_1 gene. The gene spans a distance of approximately 5.5 Kb and consists of 8 exons separated by 7 introns.

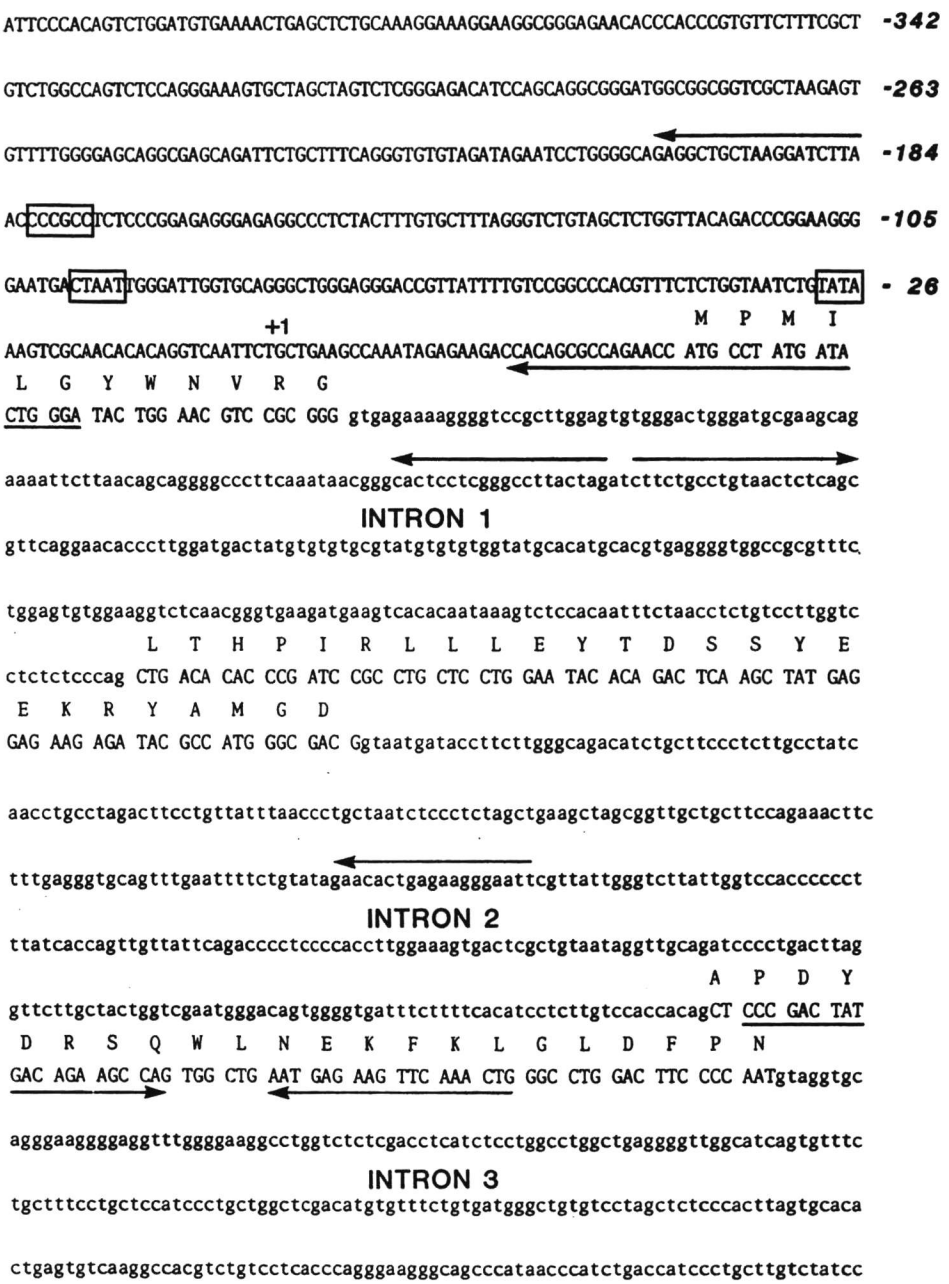


Fig. 12A. Nucleotide sequence of the glutathione S-transferase-Yb_1 gene. EcoRI fragments containing the GST-Yb_1 gene inserted into pBr325, were subjected to double-stranded nucleotide sequence analysis based on the dideoxy chain termination method. Nucleotides in the exons are denoted by uppercase letters, whereas nucleotides in the introns are denoted by lowercase letters. The TATA box and the poly(A) signal are underlined. The ATG initiation codon is enclosed in a box. The transcription initiation site is indicated by +1.

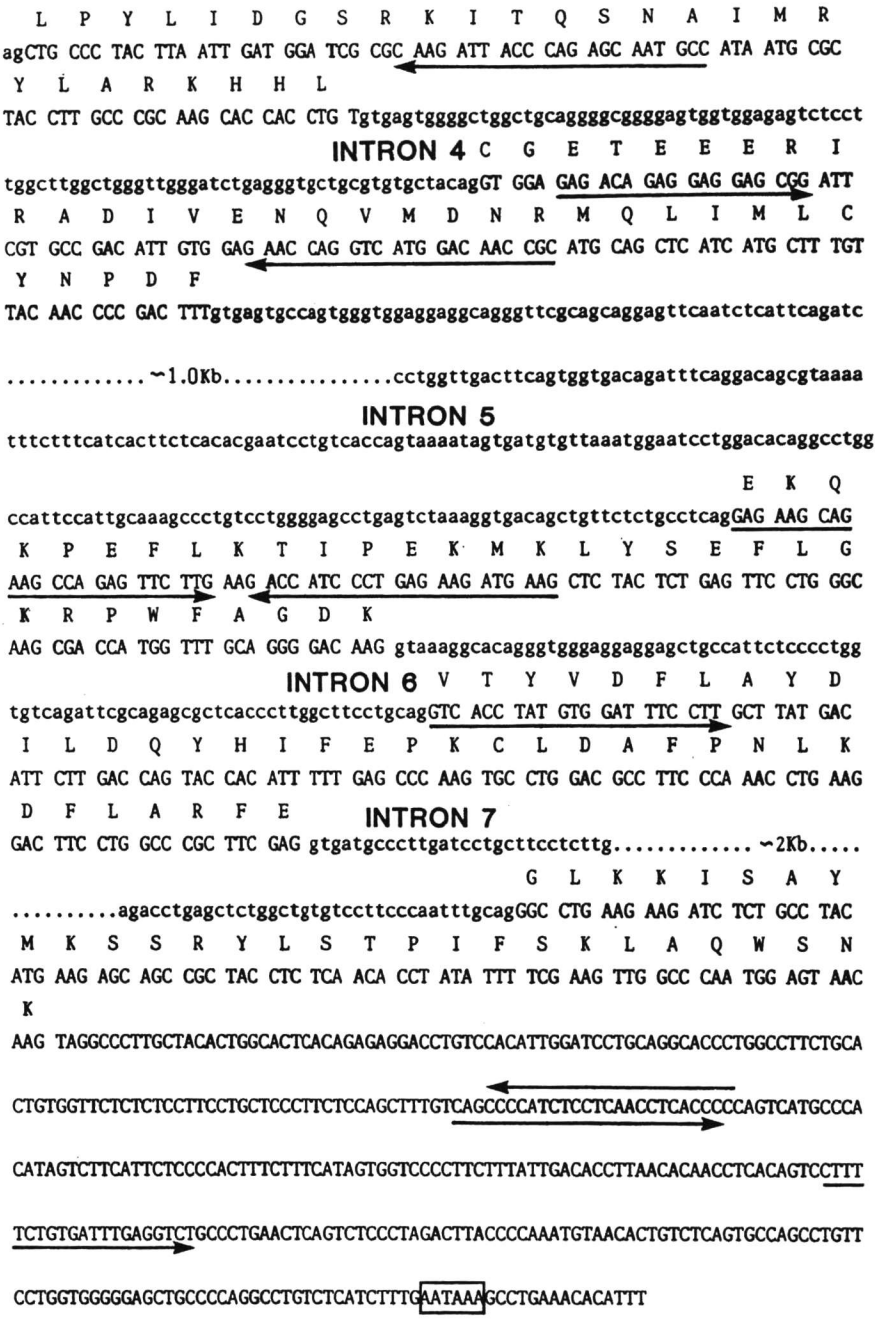


Fig. 12B. Nucleotide sequence of the glutathione S-transferase Yb_1 gene continued.

and encodes amino acids 190-218 of the Yb_1 mRNA as well as 363 bp of the 3' untranslated region of the Yb_1 mRNA.

The sizes of introns 1-4 in the glutathione S-transferase Yb_1 gene were determined by nucleotide sequence analysis, while the sizes of introns 5 and 7 were approximated using restriction mapping. The sizes of all exons and introns are listed in Table 6. All exon-intron boundaries comply with the canonical GT/AG rule (125).

The sequence of the Yb_1 structural gene is 99% homologous to the Yb_1 cDNA clone, pGTA/C44 differing by 3 nucleotides which results in 2 amino acid substitutions. Comparison of the amino acid sequence of the Yb_1 gene to the amino acid sequence of other genes from the Yb_2 gene family which have been characterized, revealed interesting homology to the Yb_2 and Yb_4 genes characterized by Lai et al. (107). Over the protein coding region, there is greater than 80% nucleotide sequence identity between the Yb_1 and Yb_2 genes (Fig. 13). Interestingly, there is a greater than 40% nucleotide sequence identity between the introns of these two genes.

Determination of transcription initiation site of Yb_1 gene

The site of transcription initiation was determined by primer extension analysis. The length of the extended primer was 54 bps, (Fig. 14) which corresponds to the guanine marked +1 in Yb_1 gene sequence (Fig. 12). An (A+T) rich region starts 29 bps upstream from the start of transcription and represents the TATA box. Also of interest, 70 bp downstream of the +1 site is the AUG translation initiation codon.

Characterization of the 5' flanking region of the glutathione S-transferase Yb_1 gene.

The genomic clone, pGTA 2.5 K, was analyzed further by DNA sequence analysis and found to contain approximately 1.9 Kb of 5' flanking sequence, containing the putative Yb_1

Table 6. Sizes of exons and introns in the glutathione S-transferase Yb_1 gene

Exon	Size (bp)	Intron	Size
1	73	1	295
2	76	2	349
3	65	3	247
4	82	4	90
5	101	5	1800
6	96	6	82
7	111	7	2000
8	458		

Table 7. Summary of intron sequence homology between Yb_1 [+] and Yb_2 genes

DNA Location	Length (bp)*	% Homology
Intron 1	294	41
Intron 2	351	49
Intron 3	247	89
Intron 4	90	70
5' Region of Intron 5	10	90
3' Region of Intron 5	57	75
Intron 6	82	74
5' Region of Intron 7	28	86

[+] Data for the Yb_2 gene was taken from Ref. 107.

*Length is relative to sequence in Yb_1 gene.

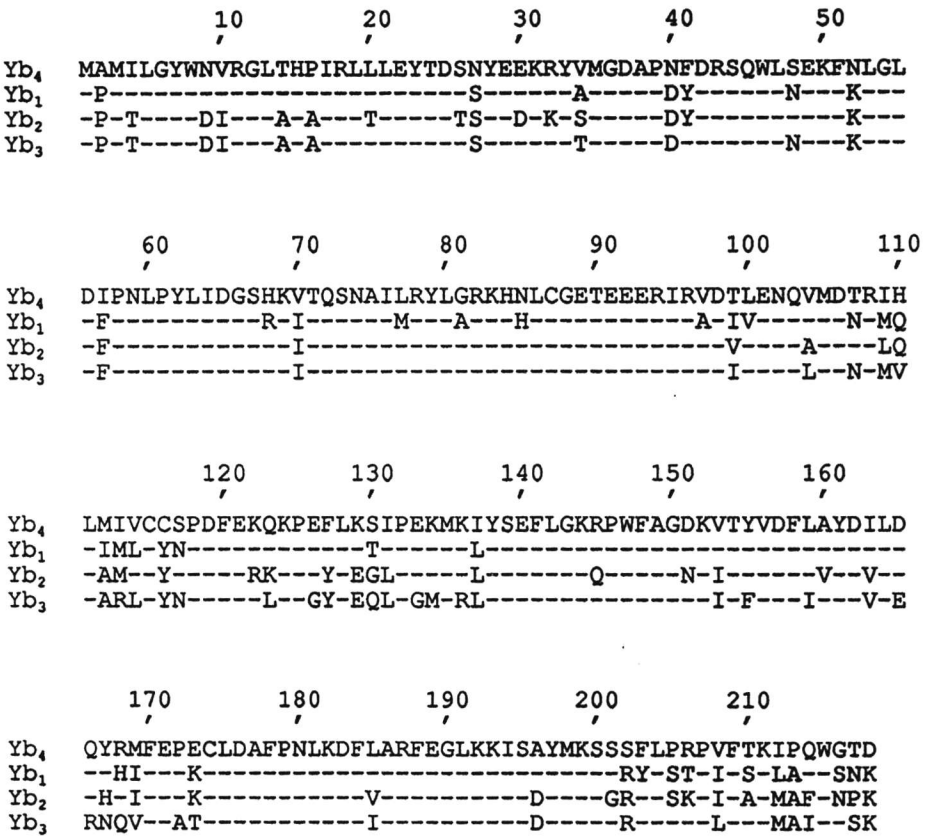


Fig. 13. Comparison of the amino acid sequence of glutathione S-transferase-Yb_1, Yb_2, Yb_3, and Yb_4 subunits.

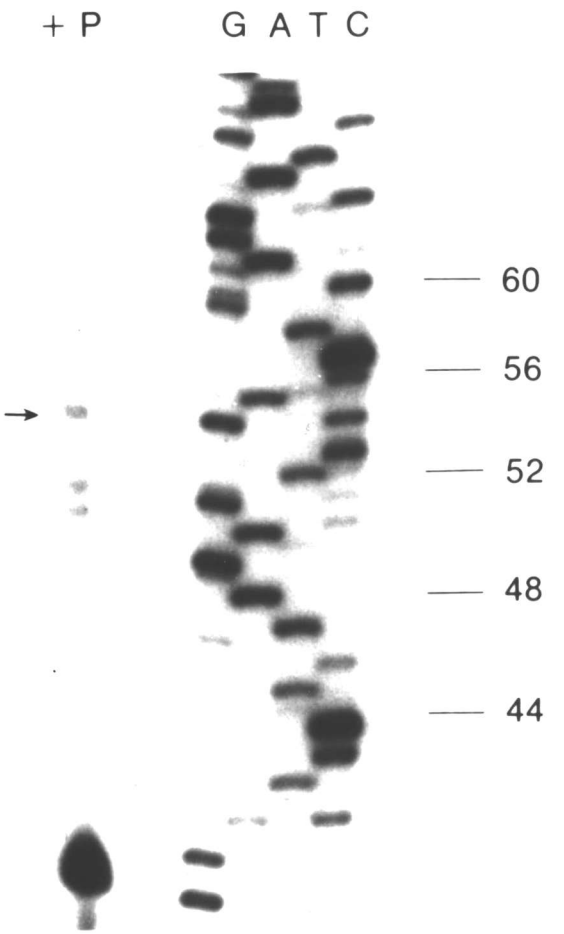


Fig. 14. Primer extension analysis. The start of transcription of the Yb_1 structural gene was determined by primer extension analysis. An oligonucleotide primer, 5'-CCC-AGT-ATC-ATA-GGC-ATG-GTT-CTG-GCG-CTG-GCG-CTG-TGG 3', which represents a 33 bp fragment complementary to the 5' region of Yb_1 mRNA was synthesized and hybridized to 30 μg of poly(A^+) RNA isolated from livers of rats treated with 3-methylcholanthrene. The primer was extended using reverse transcriptase and electrophoresed on a 12% polyacrylamide/8 M urea sequencing gel. Lane 1 contains the primer extended product. The arrow indicates the longest extended primer. Lanes 2-5 contain M13 mp19 which was used as size markers.

promoter region. The clone is represented in Fig. 15.

Plasmid DNA from pGTA 2.5 K was subjected to restriction digestion with EcoRI and BglII, thus generating a 2 Kb genomic fragment containing approximately 1.9 Kb of 5' flanking sequence and 175 bp of sequence downstream of the TATA box. The 175 bp encompass all of exon 1 and 75 bp of intron 1 (Fig. 15). The 2 Kb fragment was made blunt ended and isolated using glass bead elution as described in Methods.

Generation of Yb_1-SV_0CAT chimeric gene and transfection into mammalian cells.

The purified 2 Kb genomic fragment was inserted upstream of the structural gene encoding chloramphenicol acetyl transferase (CAT) in the pSV_0CAT reporter vector. The vector was digested with HindIII and made blunt ended prior to insertion of the Yb_1 gene fragment. The chimeric gene was transformed into SCS1 cells and tranformants were plated onto LB plates containing ampicillin. Putative positives were identified by either in situ hybridization or double restriction enzyme digestion with BamH1/Sac1. Two clones, one harboring the insert in the desired 5'---3' orientation and the other in the reverse 3'---5' orientation, were identified and subsequently introduced into Hep G2 cells (a human hepatoma derived cell line) via $CaPO_4$ mediated transfection. Results of CAT assays perfomed on cell lysates are represented in Fig. 16. No detectable CAT activity was evident for either of the Yb_1-CAT constructs, while the pSV_2CAT construct, which is under the control of the SV40 promoter and enhancer, showed appreciable activity under identical conditions. CAT activity refers to the conversion of ^{14}C-chloramphenicol [^{14}C-CAM] to its acetylated products - 1 acetyl CAM, 3 acetyl CAM and 1,3 diacetyl CAM.

Identical results, that is, no appreciable CAT activity, were obtained when the Yb_1-CAT chimeric genes were transfected into the rat hepatoma cell line, H5-6 (Fig. 17). When cells

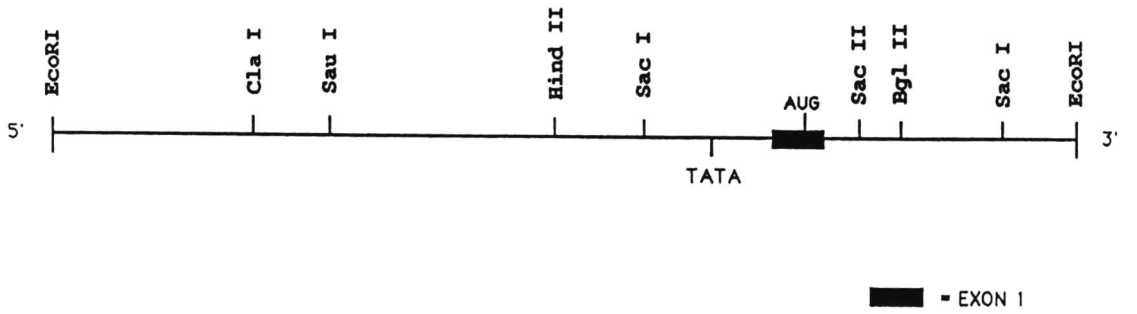


Fig. 15. Partial restriction map of pGTA 2.5K. The plasmid, pGTA 2.5K, contains approximately 1.9 kb of 5' flanking sequence and 500 bp of sequence downstream of the TATA element.

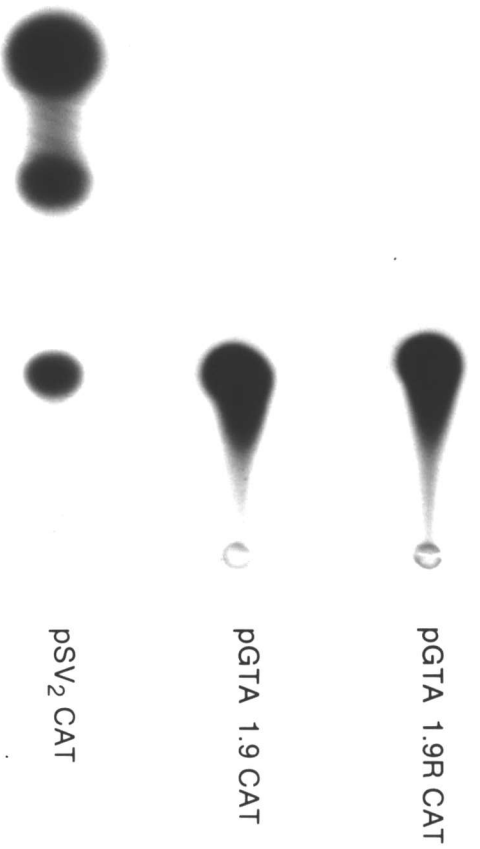


Fig. 16. Autoradiograph of CAT assay using lysed human Hep G2 cells transfected with glutathione S-transferase CAT chimeric plasmids. Transient assays were performed in human Hep G2 cells by using pSV_2CAT, pGTA 1.9 CAT, and pGTA 1.9 RCAT. All CAT assays were done in duplicate. The substrate of the reaction [^{14}C] chloramphenicol migrates closest to the origin on the TLC plate, whereas the acetylated products have a faster mobility in the solvent system.

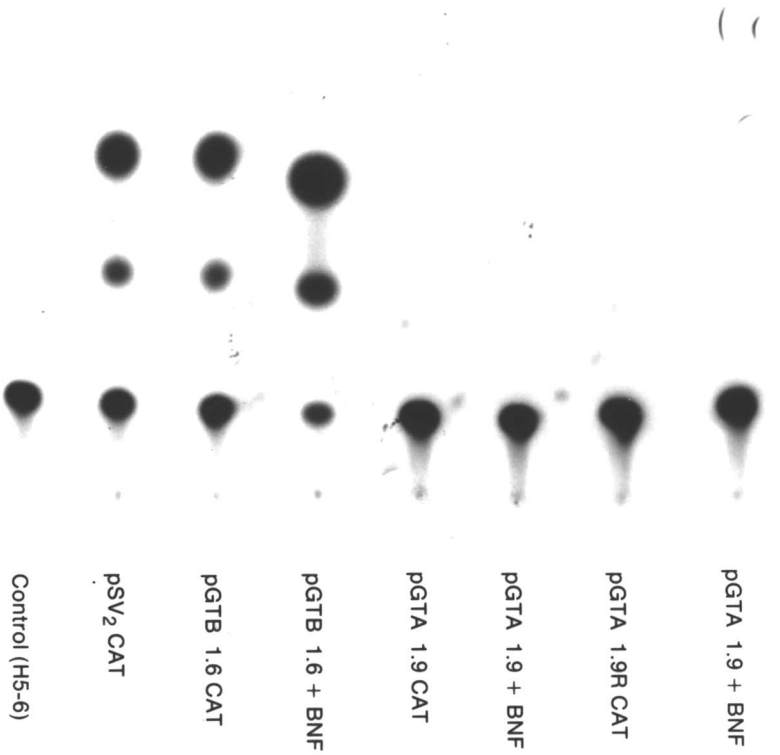


Fig. 17. Autoradiograph of CAT assay using lysed rat H5-6 cells transfected with glutathione S-transferase - chimeric plasmids. Transient assays in the presence and absence of 50 μM BNF were performed in rat H5-6 cells by using pSV_2CAT, pGTB 1.6 CAT, pGTA 1.9 CAT, and pGTA 1.9 RCAT. All assays were done in duplicate. The migration of substrate and products is described in Figure 16.

containing the glutathione S-transferase Yb_1-CAT gene were treated with β-naphthoflavone, a compound known to induce the glutathione S-transferase-Ya CAT gene in cell culture, no activity was detected (Fig. 17).

Construction of Yb_1-CAT chimeric genes using Bal-31 exonuclease and the polymerase chain reaction.

Careful examination of the nucleotide sequence of the Yb_1 2 Kb structural gene fragment, which was previously inserted into pSV_0CAT, revealed that the fragment contained all of exon 1 which contained two initiation codons (AUG). The lack of CAT activity of the initial construct could be explained if translation were initiated at the start site of the Yb_1 gene thereby resulting in a frameshift and synthesis of a non-functional protein.

Two different techniques were utilized to generate Yb_1-CAT chimeric genes which did not contain the AUG initiation codon. The first technique is a novel procedure employing in vitro amplification of DNA, using Taq polymerase to amplify the Yb_1 structural gene fragment. The previously constructed Yb_1-CAT chimeric gene was used as the template and oligonucleotide complementary to the 5' and desired 3' end were designed and used in the amplification to generate pGTA 1.7 CAT. A schematic depiction of this clone is represented in Fig. 18.

The second technique involved exonuclease digestion of the initial construct Yb_1 CAT with Bal-31 after restriction digestion of the plasmid DNA with SacII. A unique SacII site is located 75 bp downstream of the transcription initiation site (Fig. 15). After digestion of the plasmid with SacII and subjection to Bal-31 digestion, the linearized fragment was made blunt ended using Klenow and re-ligated in presence of T_4 ligase. The chimeric genes were transformed into SCS1 cells as previously described and transformants were plated on LB

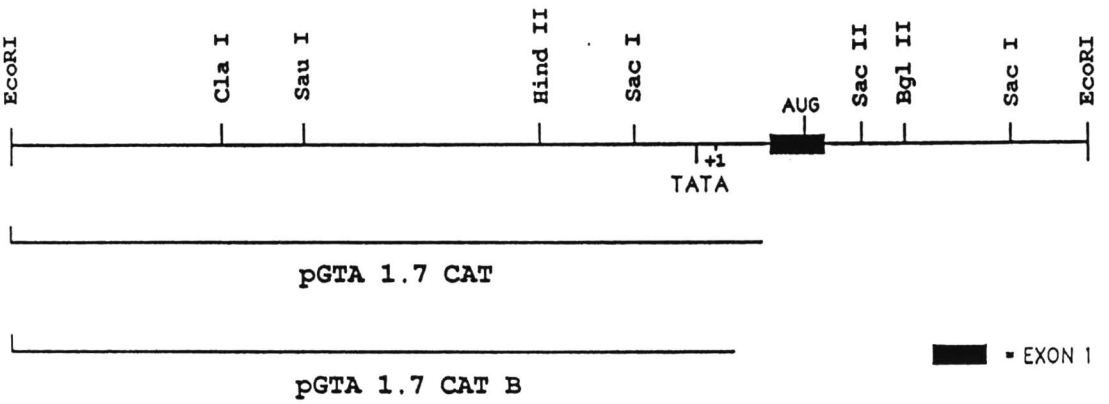


Fig. 18. Schematic diagram of pGTA1.9CAT, pGTA1.7CAT and pGTA1.7CATB. The chimeric plasmids were constructed as described in the Discussion. THe plasmids pGTA1.9CAT, pGTA1.7CAT, and pGTA1.7CATB were constructed by EcoRI/BglII digestion of pGTA2.5K, by PCR, and by Bal 31 treatment, respectively.

plates containing ampicillin. A schematic depiction of this clone, pGTA 1.7 CATB, is also included in Fig. 18.

Positive clones, containing the Yb_1 gene fragment in the proper orientation, as determined by double restriction digestion with BamHI and SacI, were grown on a large scale. Plasmid DNA was transfected into Hep G2 cells as described previously. The results of CAT assays of the cell lysates are presented in Fig. 19 and 20. This data demonstrated that the Yb_1 structural gene fragment contained a functional promoter and that comparable CAT activity resulted from generation of the PCR clone and from Bal-31 exonuclease treatment. The question of transcriptional activity of the Yb_1 promoter was answered and another issue addressed - that is the induction of the Yb_1 gene activity in response to xenobiotic administration. Cells which had been transfected with the active Yb_1-CAT constructs were treated with 3MC and activity of the cell lysates was determined. The results, depicted in Fig. 19 and 20 demonstrated that the Yb_1 gene activity is not induced in Hep G2 cells in response to 3MC administration. The activity of the Yb_1-CAT chimeric gene was not elevated in response to Pb treatment of either Hep G2 or H5-6 cells (data not shown). These data suggested that regulatory element(s) that confer 3MC inducibility are lacking from the first 1700 bp of the Yb_1 gene. The studies of Pb induction of the Yb_1 gene activity await the availability of a Pb responsive cell line.

Localization of the element required for basal expression of Yb_1 gene.

A series of deletions of the pGTA 1.7 CAT gene fragment were constructed using PCR and inserted into pSV_0CAT. The deletions contained 1200, 1000, 760, 610, 550, 510, 480, 280, 120, and 50 bps of 5' flanking sequence (Fig. 21). The smallest mutant, pGTA 0CAT contains the TATA element and sequences downstream of it. The 3' end of each mutant is

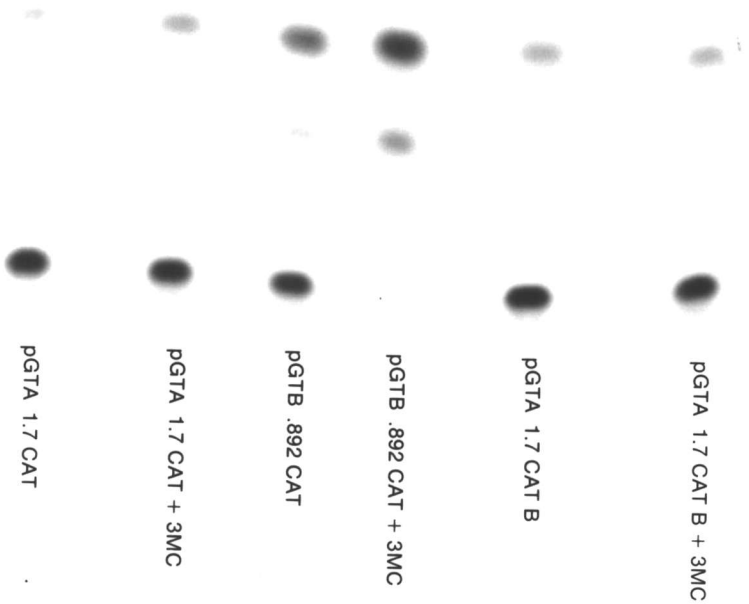


Fig. 19. Autoradiograph of CAT assay using lysed human Hep G2 cells transfected with glutathione S-transferase-CAT chimeric plasmids. Transient assays in the presence and absence of 3MC were performed in human Hep G2 cells by using pGTA 1.7 CAT, pGTB .892 CAT, and pGTA 1.7 CAT B. All assays were done in duplicate. The migration of substrate and products is described in Figure 16.

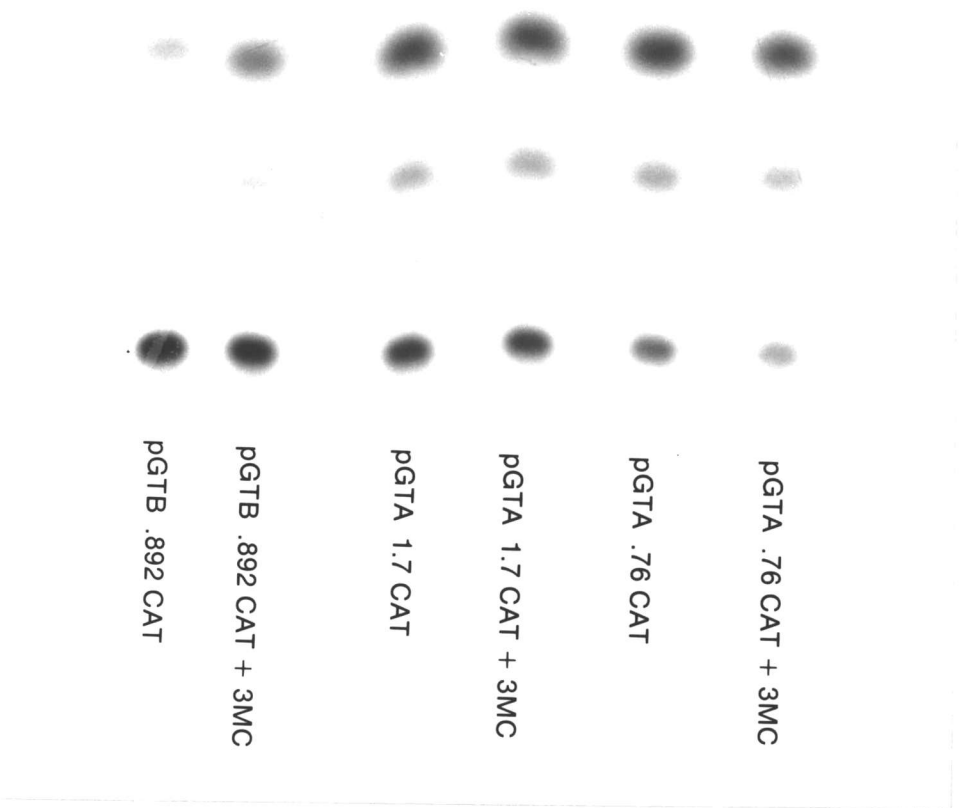


Fig. 20. Autoradiograph of CAT assay using lysed human Hep G2 cells transfected with glutathione S-transferase-CAT chimeric plasmids. Transient assays in the presence and absence of 3MC were performed in human Hep G2 cells by using pGTB .892 CAT, pGTA 1.7 CAT, and pGTA .76 CAT. All assays were done in duplicate. The migration of substrate and products is described in Figure 16.

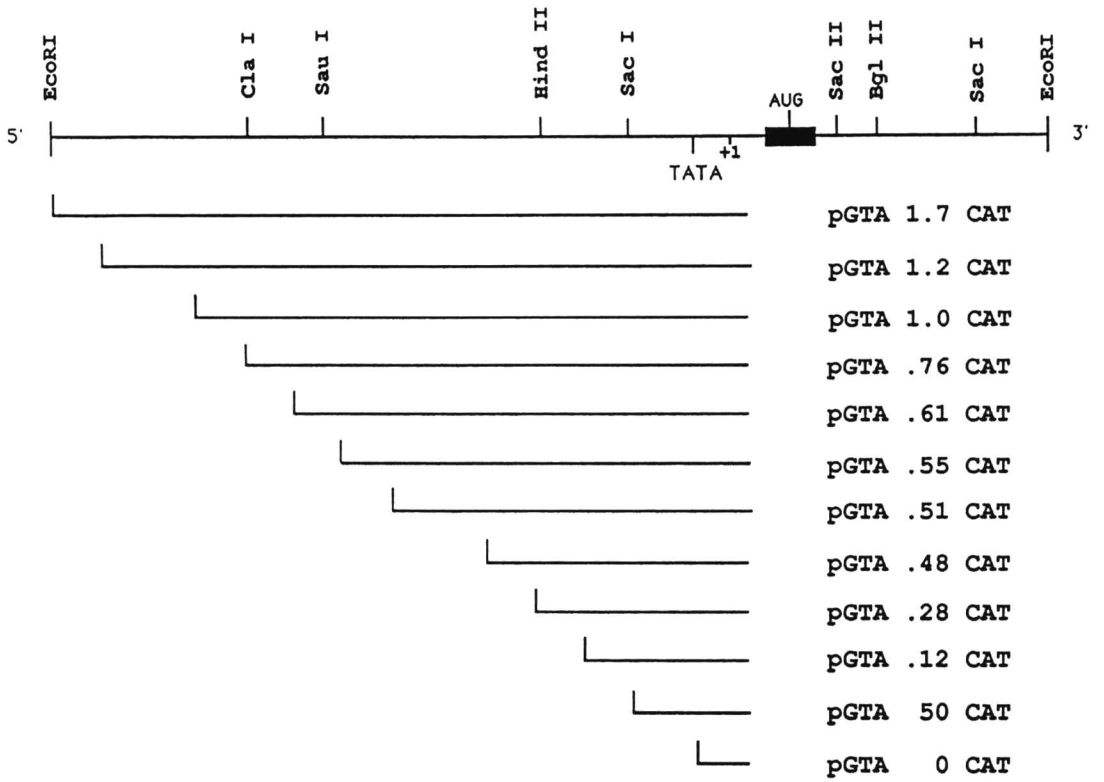


Fig. 21. Schematic diagram of PCR generated Yb_1-CAT chimeric plasmids.

identical to the 3' end of the initial PCR construct. The 5' and 3' ends are highlighted in Fig. 22. These mutants were transfected into Hep G2 cells and the activity of the cell lysates was quantitated using CAT assays. The results of the CAT assays are presented in Fig. 23-27.

All transfections were performed a minimum of 3 times with at least two different preparations of DNA. These data suggested that while marginal differences exist in the activity of the first 10 deletions, there is a distinct decrease (60-70%) in transcriptional activity when the sequence up to 50 bp upstream of the TATA box is removed. This suggested that this region contains the element(s) responsible for basal expression of the Yb_1 gene (Fig. 26-27). Contained within this region are the TATA element, the transcription start site, and 50 bp upstream of the TATA element.

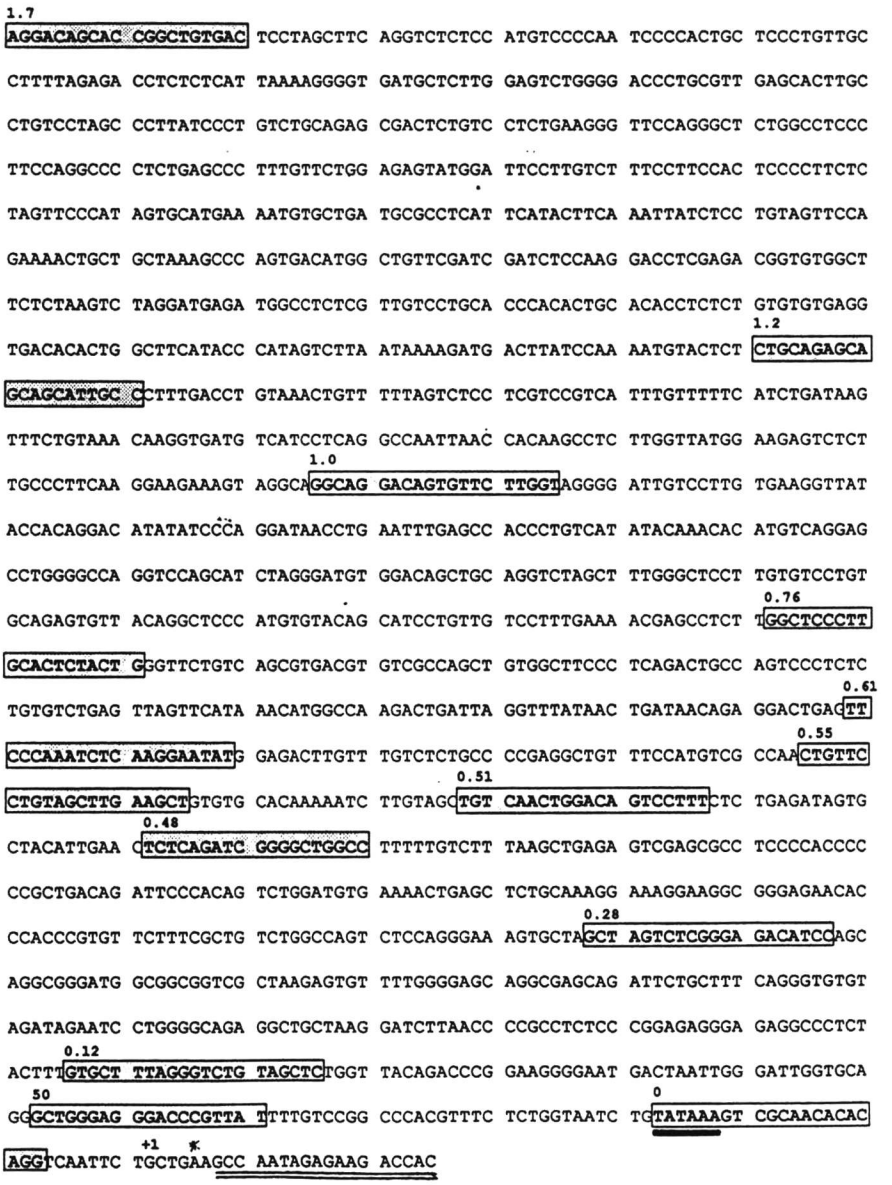


Fig. 22. Nucleotide sequence of 5' flanking region of the GST-Yb_1 gene. The boxes represent the sequence of oligonucleotides used to generate the Yb_1-CAT chimeric constructs by the polymerase chain reaction. The TATA box is underlined in bold print. The 3' end of all PCR generated clones is double underscored. The asterisk denotes the 3' end of pGTA 1.7 CATB.

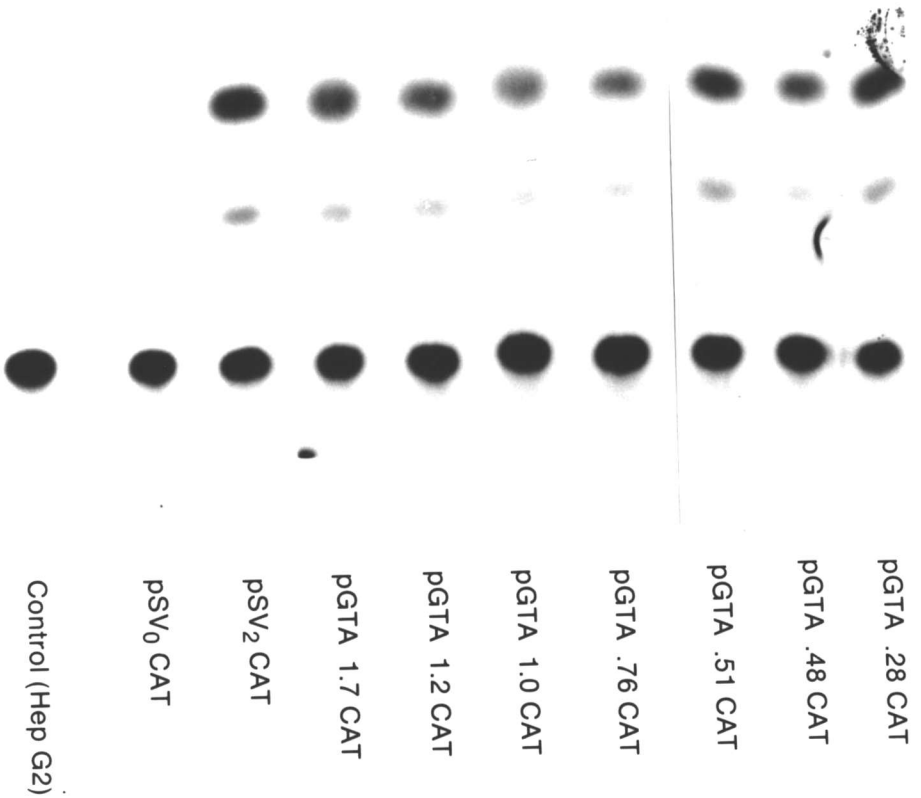


Fig. 23. Autoradiograph of CAT assay using lysed human Hep G2 cells transfected with glutathione S-transferase-CAT chimeric plasmids. Transient assays were performed in human Hep G2 cells by using no plasmid, pSV_0CAT, pSV_2CAT, pGTA 1.7 CAT, pGTA 1.2 CAT, pGTA 1.0 CAT, pGTA .76 CAT, pGTA .51 CAT, pGTA .48 CAT, and pGTA .28 CAT. The migration of substrate and products is described in Figure 16.

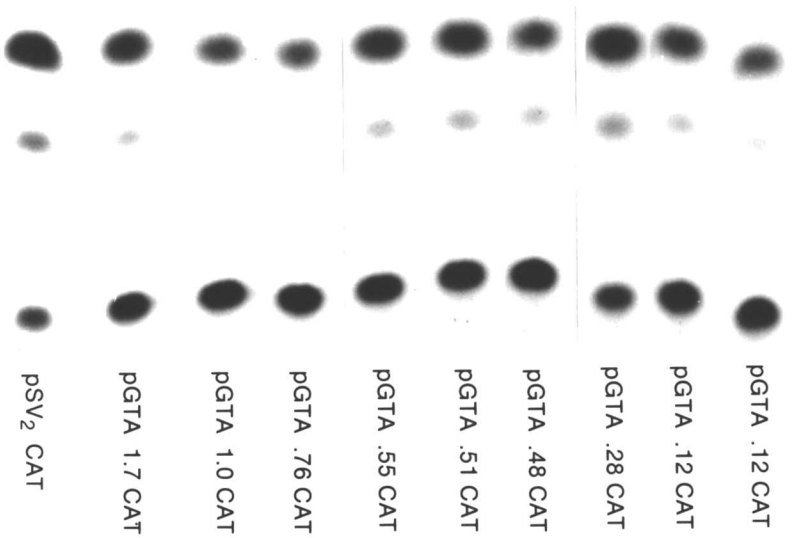


Fig. 24. Autoradiograph of CAT assay using lysed human Hep G2 cells transfected with glutathione S-transferase-CAT chimeric plasmids. Transient assays were performed in human Hep G2 cells by using pSV_2CAT, pGTA 1.7 CAT, pGTA 1.0 CAT, pGTA .76 CAT, pGTA .55 CAT, pGTA .51 CAT, pGTA .48 CAT, pGTA .28 CAT, and pGTA .12 CAT. Assays were done in triplicate. The migration of substrate and products is described in Figure 16.

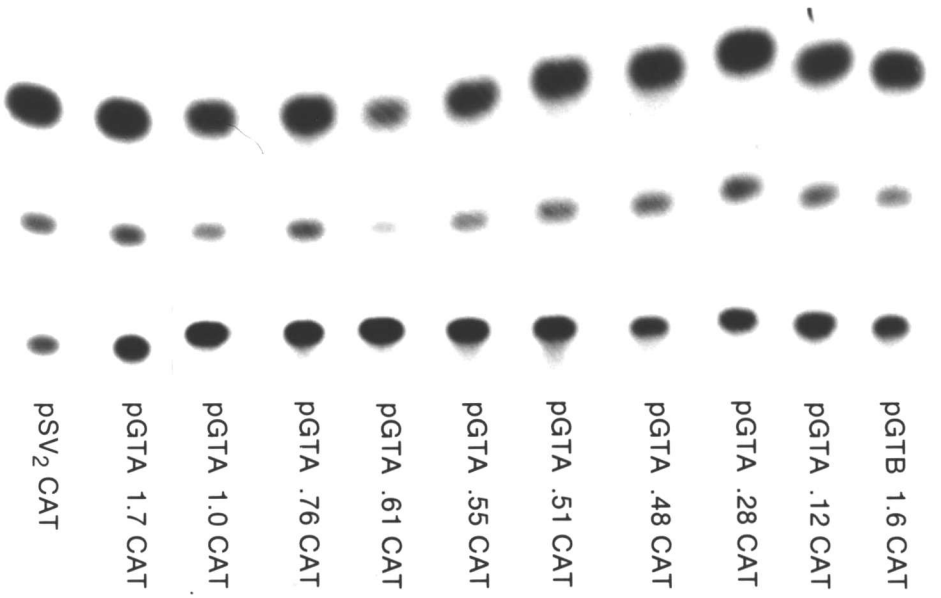


Fig. 25. Autoradiograph of CAT assay using lysed human Hep G2 cells transfected with glutathione S-transferase-CAT chimeric plasmids. Transient assays were performed in human Hep G2 cells by using pSV_2CAT, pGTA 1.7 CAT, pGTA 1.0 CAT, pGTA .76 CAT, pGTA .61 CAT, pGTA .55 CAT, pGTA .51 CAT, pGTA .48 CAT, pGTA .28 CAT, pGTA .12 CAT and pGTB 1.6 CAT. All assays were performed in triplicate. The migration of substrate and products is described in Figure 16.

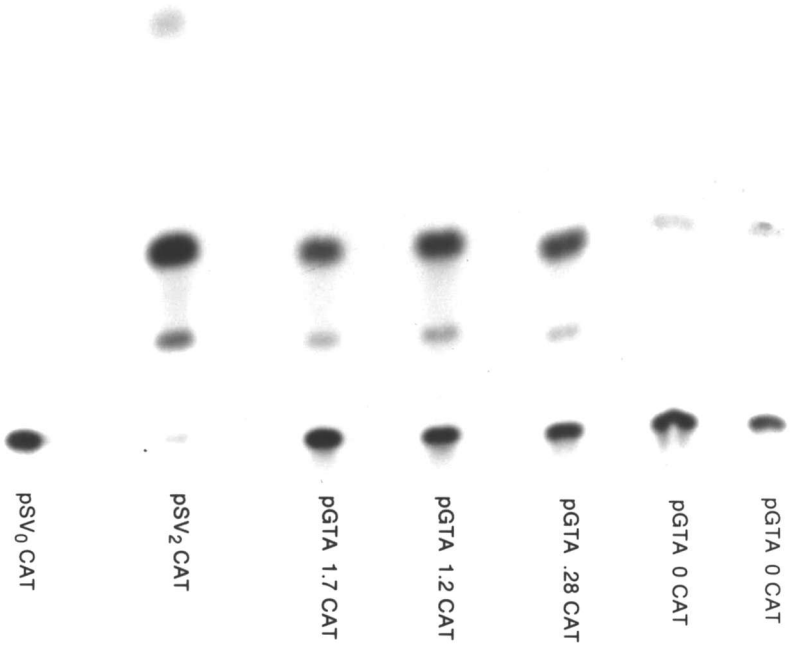


Fig. 26. Autoradiograph of CAT assay using lysed human Hep G2 cells transfected with glutathione S-transferase chimeric plasmids. Transient assays were performed in human Hep G2 cells by using pSV_0CAT, pSV_2CAT, pGTA 1.7 CAT, pGTA 1.2 CAT, pGTA .28 CAT, and pGTA 0 CAT. All assays were done in triplicate. The migration of substrate and products is described in Figure 16.

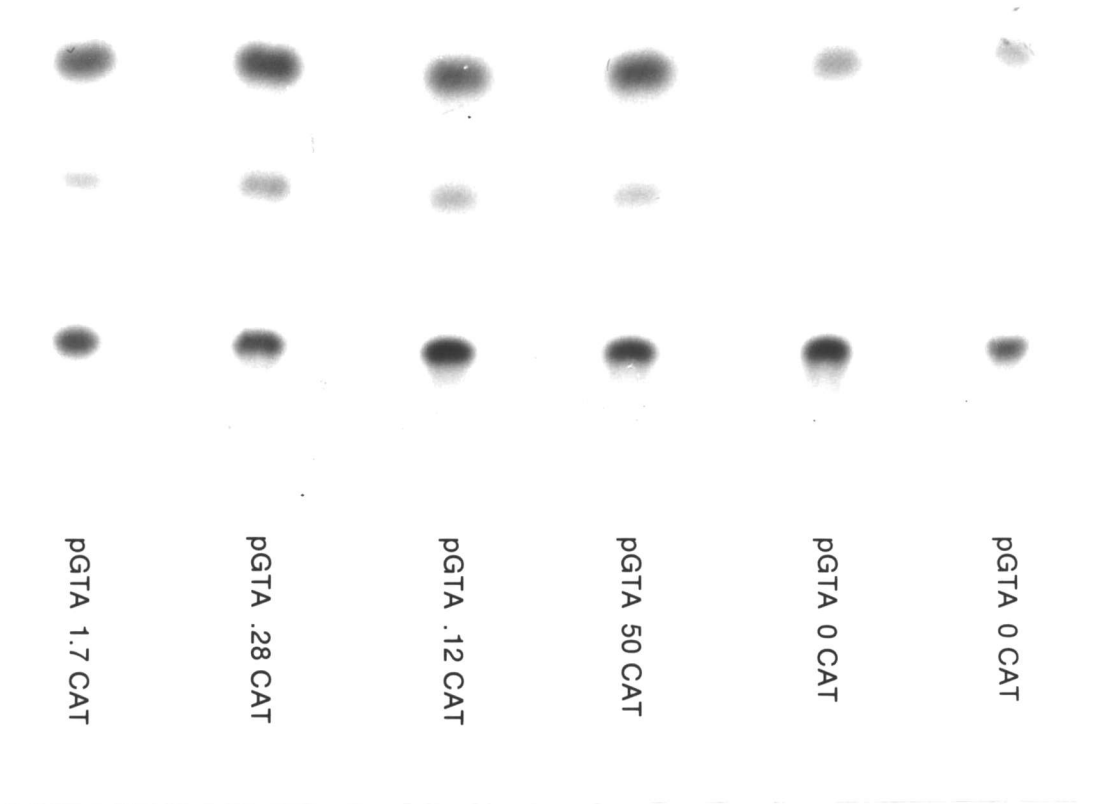


Fig. 27. Autoradiograph of CAT assays using lysed human Hep G2 cells transfected with glutathione S-transferase-CAT chimeric plasmids. Transient assays were performed in human Hep G2 cells by using pGTA 1.7 CAT, pGTA .28 CAT, pGTA .12 CAT, pGTA 50 CAT, and pGTA 0 CAT. Assays were done in triplicate. The migration of substrate and products is described in Figure 16.

CONCLUSION

Many pathological conditions, such as cancer, are caused by exposure to toxic agents. It is therefore important to understand the toxic effects and metabolism of compounds to which living beings are exposed. In the body, foreign compounds usually undergo chemical transformations, such as oxidations, reductions, hydrolyses, and syntheses (conjugations). Most often, transformations of foreign compounds result in less active products. The tranformations are generally catalyzed by enzymes, many of which occur in the liver. One group of such enzymes is the glutathione S-transferases. The transferases are called detoxicating enzymes since by catalyzing the conjugation with glutathione and rendering nonpolar foreign molecules more hydrophilic, they initiate the first stage of the mercapturic acid pathway for elimination of foreign compounds from the body (68).

It has been well documented that the transferases, as well as other drug metabolizing enzymes such as NADH cytochrome P-450 reductase and epoxide hydrolase, are regulated at the transcriptional level, in response to xenobiotic administration. Recent work on the transferases has focused on the regulation of the rat and mouse Ya genes and on the Yp gene.

The evolution and regulation of the Yb genes constitute the focus on the work described in this text. A full-length Yb_1 gene was isolated from a rat liver genomic library using the Yb_1 cDNA clone, pGTA/C44, as a probe. Nucleotide sequence analysis revealed that the gene spans a distance of approximately 5.5 Kb and is comprised of eight exons separated by seven introns. The nucleotide sequence of the Yb_1 structural gene is 99% homologous to the Yb_1 cDNA clone, pGTA/C44. The characterization of the Yb_1 gene provides the final evidence that the gene is a member of a distinct gene family.

A comparison of the nucleotide sequence of the Yb_1 and Yb_2 (92) genes revealed significant homology in the 5' end of the coding region as well as in the middle; the 3' ends are

divergent. Interestingly, there is also significant homology throughout the introns of these two genes. This data suggests that gene conversion, a nonreciprocal recombination event, may have played a role in the evolution of the two genes.

Isolation and characterization of the gene has facilitated an analysis of the 5' flanking region of the gene. A DNA fragment containing approximately 1.7 Kb of upstream sequence (and various deletions of the sequence) was inserted into pSV_0CAT, a reporter vector which lacks a promoter and contains the structural gene which encodes chloramphenicol acetyl transferase. The chimeric genes were introduced into mammalian cells, which lack endogenous CAT activity, and CAT activity was monitored using thin layer chromatography. The data suggests that the regulatory elements responsible for basal expression of the Yb_1 gene are contained within an 80 bp region of sequence upstream of the transcription start site. This region contains a TATA element, transcription start site, and 50 bp of sequence upstream of the TATA element. A deletion mutant lacking this region consistently yielded CAT activities which were approximately 30% of the activity obtained using chimeric genes containing this region.

Several labs have demonstrated that the glutathione S-transferase Ya and Yb mRNAs are elevated in the liver of rats administered 3MC. No elevation in Yb_1 promoter activity was detected when rat or human hepatoma cells which had been transfected with the Yb_1 promoter-CAT chimeric genes, were treated with this xenobiotic. When cells which had been transfected with the glutathione S transferase Ya promoter were treated with 3MC, Ya promoter activity was elevated 4-5 fold. This data suggests that the the regulatory element which confers 3MC inducibility is absent from the first 1700 bp of the Yb_1 gene. The task of identifying the regulatory element(s) responsible for activation of the Yb_1 gene activity in

response to phenobarbital adminstration was hindered by the lack of availability of a phenobarital-responsive cell line. The use of primary hepatocyte cultures may be necessary to examine phenobarbital induction.

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